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Methods: Altogether 43 AS and 44 healthy controls were included in the study. Anti-MCV and anti-hsp65 were determined in sera by commercial and in-house ELISA, respectively. Serum autoantibody levels were correlated with ESR, CRP, HLA-B27 status, smoking habits, pain intensity, BASDAI, BASFI and BASMI indices.
Results: Patients with AS had significantly 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) ($p<0.01$). Sixteen 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). The mean anti-hsp65 concentration in AS sera was 124.8 AU/ml (range: 27.2-1000 AU/ml), while controls exerted significantly lower anti-hsp65 levels (mean: 51.8 AU/ml; range: 22.5-88.5 AU/ml) ($p<0.001$). Correlation analysis revealed that both anti-MCV positivity ($r=0.613$; $p=0.012$) and absolute serum anti-MCV levels ($r=0,553$; $p=0.021$) correlated with anti-hsp65 levels. Anti-MCV positivity also correlated with ESR ($r= 0.437$; $p=0.03$).
Conclusions: Anti-MCV and anti-hsp65 may be novel biomarkers in AS.

Anti-mutated citrullinated vimentin (anti-MCV) and anti-65 kDa heat shock protein (anti-hsp65): new biomarkers in ankylosing spondylitis

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Running head: Anti-MCV and anti-hsp65 in ankylosing spondylitis

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List of abbreviations

ACPA	anti-citrullinated protein antibodies
AS	ankylosing spondylitis
BASDAI	Bath ankylosing spondylitis disease activity index
BASFI	Bath ankylosing spondylitis functional index
BASMI	Bath ankylosing spondylitis metric index
CCP	cyclic citrullinated peptide
CF	citrullinated fibrinogen
CRP	C reactive protein
CV	citrullinated vimentin
DMARD	disease-modifying antirheumatic drug
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
HLA	human leukocyte antigen
Hsp	heat shock protein
IgG	immunoglobulin G
MCV	mutated citrullinated vimentin
MRI	magnetic resonance imaging
NSAID	nonsteroidal antiinflammatory drug
PsA	psoriatic arthritis
RA	rheumatoid arthritis
Sa	Savoie
SpA	spondylarthritis (spondylarthropathy)
TNF	tumor necrosis factor
VAS	visual analogue scale

Abstract

Introduction: Citrullination as well as anti-citrullinated protein/peptide antibodies (ACPA) have been implicated in the pathogenesis of rheumatoid arthritis (RA). While ACPAs are specific and sensitive markers for RA, there have been hardly any reports regarding ACPAs in ankylosing spondylitis (AS). The possible role of antibodies to Mycobacterial 65 kDa heat shock protein (hsp65) has not been characterized in AS. As new laboratory biomarkers of AS are needed, we investigated the prevalence of anti-mutated citrullinated vimentin (MCV) and anti-hsp65 antibodies in AS.

Methods: Altogether 43 AS and 44 healthy controls were included in the study. Anti-MCV and anti-hsp65 were determined in sera by commercial and in-house ELISA, respectively. Serum autoantibody levels were correlated with ESR, CRP, HLA-B27 status, smoking habits, pain intensity, BASDAI, BASFI and BASMI indices.

*Results: P*atients with AS had significantly 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) ($p<0.01$). Sixteen 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). The mean anti-hsp65 concentration in AS sera was 124.8 AU/ml (range: 27.2-1000 AU/ml), while controls exerted significantly lower anti-hsp65 levels (mean: 51.8 AU/ml; range: 22.5-88.5 AU/ml) ($p<0.001$). Correlation analysis revealed that both anti-MCV positivity ($r=0.613$; $p=0.012$) and absolute serum anti-MCV levels ($r=0,553$; $p=0.021$) correlated with anti-hsp65 levels. Anti-MCV positivity also correlated with ESR ($r= 0.437$; $p=0.03$).

Conclusions: Anti-MCV and anti-hsp65 may be novel biomarkers in AS.

Introduction

Ankylosing spondylitis (AS) is an inflammatory rheumatic disease that may lead to functional impairment of the spine and peripheral joints. Genetic predisposition and environmental factors have been implicated in the pathogenesis of AS [1, 2]. With new and very effective therapeutic approaches, such as biologics becoming available, it is imperative to recognize and treat AS as early as possible in order to prevent disability. In early stages, spinal and sacroiliac MRI may be a useful radiological tool for the diagnosis and follow-up of AS [3, 4], however, there is a need for laboratory biomarkers. While anti-citrullinated protein/peptide antibodies (ACPA) have become rather specific and sensitive diagnostic tools in rheumatoid arthritis (RA) [5-10], such autoantibodies have not yet been identified in AS.

ACPAs including anti-cyclic citrullinated peptide (CCP), anti-citrullinated vimentin (CF), anti-citrullinated fibrinogen (CF), anti-citrullinated α enolase and some others have been implicated in the pathogenesis and outcome of RA [6-8, 11-19]. 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 [8, 11, 19-21].

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 [22]. In order to detect antibodies to CV, an ELISA system was developed that contains genetically modified, mutated citrullinated vimentin (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 [7, 11, 18, 23-26].

There have been few data on the possible associations of ACPA with spondyloarthropathies (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 [27]. In a recent study, 15% of PsA and 14% of AS patients were positive for anti-MCV [28].

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 [29-31]. Regarding AS, Mycobacteria have been implicated in the pathogenesis of the disease [32, 33], 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 [34]. No other reports on anti-hsp65 in relation to AS have become available.

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 the present study, 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 with disease duration, erythrocyte sedimentation rate (ESR), C reactive protein (CRP), HLA-B27 status, BASDAI, BASFI, BASMI, and pain on visual analogue scale (VAS). As ACPA production has been associated with smoking in RA [21], we also correlated antibody production with the smoking habits of AS patients.

Patients and methods

Patients and controls

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 [35]. 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). Most patients (37/43, 86%) received nonsteroidal antiiflammatory drugs (NSAID). 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 biologics. None of the AS patients were genetically related or had any type of arthritis in their family.

Regarding clinical assessments, AS disease activity, functional capacity and mobility were tested by obtaining BASDAI, BASFI and BASMI, respectively. Pain intensity was recorded on a VAS scale. ESR (mm/h) was assessed by the Westergren method. Serum CRP levels (mg/l) were measured by quantitative turbidimetry (Cobas Mira Plus, Roche), using CRP reagents (Dialab, Austria). 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).

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 cardiovascular, 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 [36] in RA (≥ 20 pack-years). Cumulative tobacco intake was calculated by multiplying the mean daily intake by the duration of consumption in years.

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.

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 [7]. 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 [37]. Data obtained as optical density values were calculated as arbitrary unit per ml (AU/ml) values related to standard.

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.

Results

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 1).

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).

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 2).

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).

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).

Discussion

ACPAs are considered to be specific and sensitive diagnostic markers of RA [5-7, 38]. 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 [28] 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 our present study, 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 [30, 33, 34]. There has been only one study reporting non-significant elevation of anti-hsp65 in 19 out of 59 AS patients [34], however anti-hsp65 was not assessed in association with other clinical or laboratory parameters. Here we also found significantly elevated serum anti-hsp65 levels in AS. Our results are somewhat different from those published by McLean et al [34] as in their study, the elevation of anti-hsp65 in AS compared to controls was not statistically significant.

Interestingly, 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 [32, 33]. Among citrullinated proteins, citrullinated vimentin has also been detected in the synovial tissues of SpA, as well as RA patients [39]. 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 [32, 33, 39]. It is not clear whether there would be a direct cross-reactivity between anti-MCV and anti-hsp65 antibodies in AS.

Conclusion

In conclusion, although the exact role of anti-MCV and anti- Mycobacterial hsp65 autoantibodies in AS remains to be further characterized, our results suggest a possible novel role of these autoantibodies in AS. Moreover, as anti-hsp65 and anti-MCV exerted positive correlation with each other, these two, very different antibodies may have a role in the pathogenesis of AS and they may also serve as useful antibody biomarkers.

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Figure legends

Figure 1. Scatter plot showing anti-MCV levels in the sera of AS patients and controls.

Figure 2. Scatter plot showing anti-hsp65 levels in the sera of AS patients and controls.

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Table 1 Description of the AS population

Variable (unit)	Median±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	-
Psoriasis (n; %)	3 (7)	-
Uveitis (n; %)	12 (28)	-
Inflammatory bowel disease (n; %)	2 (5)	-
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
Active disease (BASDAI>40; %)	32.6	-
BASDAI (mm)	50.4±19.1	19-80
BASFI	45.4±11.8	
BASMI	45.4±11.8	
Current NSAID therapy (%)	86%	-
Current DMARD therapy (%)	14%	-
Current anti-TNF therapy (%)	65%	-

*Response to Reviewers

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DEBRECENI EGYETEM
ORVOS- ÉS EGÉSZSÉGTUDOMÁNYI CENTRUM
Belgyógyászati Intézet
REUMATOLÓGIAI TANSZÉK
Tanszékvezető: Prof. Dr. Szekanecz Zoltán egyetemi tanár

UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTRE
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Professor Marie-Christophe Boissier
Editor-in-Chief
Joint Bone Spine

Re: BONSOI-D-10-00454

Dear Professor Boissier,

We wish to thank You and the Reviewers for commenting our manuscript.

We address all isuees as follows:

1. We thank the reviewer for finding our paper interesting. We picked anti-MCV as there have been numerous papers on anti-CCP and we did not want to repeat those studies. Citrullinated vimentin has been detected by others in arthritis synoviual tissues (see Tilleman et al), but anti-CV has not yet been studied. The aim of our study was to find NEW biomarkers for AS, and anti-MCV is really novel. We did not test anti-CCP on this cohort, so we are unable to present such data.
2. We agree that the study design is not perfect. While anti-MCV was assessed by a commercial ELISA so we did not have any limitation, anti-hsp65 was tested by a home-made ELISA using a commercial antibody. Therefore we could not test as many subjects as we wished. Therefore , 44 fully healthy controls were used for anti-MCV and only 11 other low-back-pain controls for anti-hsp65. In addition, the two assessment swqere not performed at the same time. The anti-hsp65 assay was performed already in 2009, while the anti-MCV study was done in 2010.
3. Now we enclosed two figures that are scatter plots to show oriuginal anti-hsp and anti-MCV results. Accordingly, we deleted Table 2 and included figure legends for the two new figs.
4. None of the patients were genetically related and none of them had RA or other types of arthritis in their family. This information is now included int he methodology section.
5. Peripheral vs axial AS classification was determined during the history of the disease. There was no difference between autoantibody levels in axial vs peripheral AS patients. Data on extraarticular manifestations are now listed in Table 1. Three patients had psoriasis, 12 had uveitis and 2 had IBD. Although these numbers are relatively small, we correlated them with the antibodies and found no correlations. This is now mentioned in the Results section.

We hope that we satisfactorily addressed the issues raised by the Reviewer. We again thank you for your work and we hope that our manuscript is now acceptable for publication.

Sincerely yours,

Prof. Zoltán Szekanecz, MD, PhD

Figure(s)

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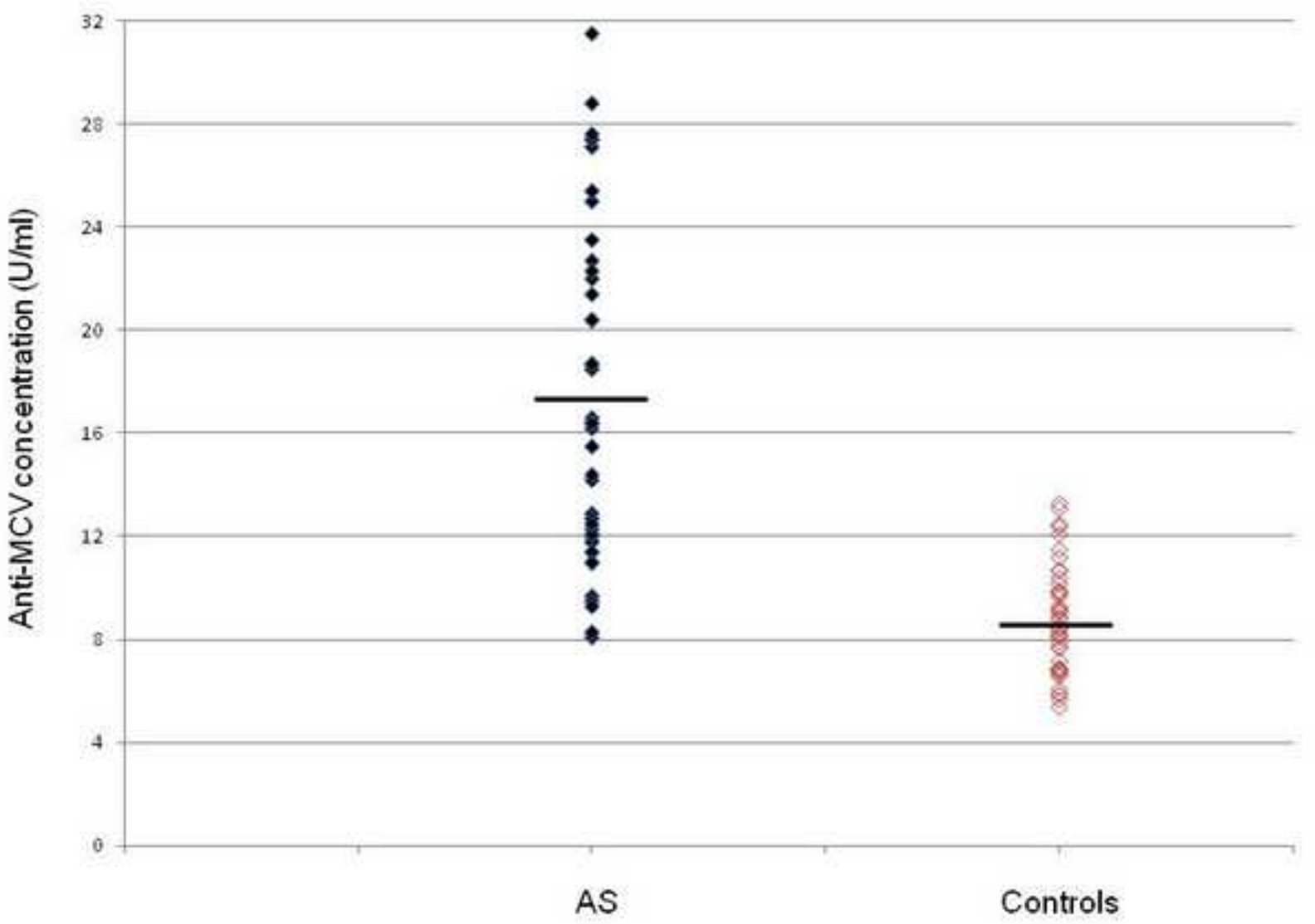

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