Clinical Medical Reviews and Case Reports

Review Article: Open Access

Outcomes of Influence of Dimethylarginine Derivats, PAD Isoenzymes, or both as Hallmark on Citrullinisation Process on Autoantigens ACPA/Anti CCP Antibodies overall for Diagnosis in Rheumatoid Arthritis

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Abstract

Introduction: When establishing new diagnostic method for detection of the endothelial dysfunction in Rheumathoid Arthritis (RA), it is necessary to compare the diagnostic values with other laboratory variables.

Aim: To Assess Asymmetric Dimethylarginine (ADMA) in RA and study its relation to ACPA and different clinical and laboratory parameters of disease activity in early non treated RA patients. Also, to determine the association between the asymmetric dimethylarginine (ADMA) and anti-cyclic citrullinated peptide antibodies (ACPA, Anti-CCP$_2$) of the second generation in RA and to explain their connection in the process of citrulination of autoantigens in RA. Also, to determine the association between ADMA and the acute phase reactants: Rheumatoid factor (RF), C-reactive protein (CRP), as well as the index of disease intensity (DAS$_{28}$) in early diagnosis in non-treated RA patients and to determine whether the amount of ADMA changes in the course of the evolution of disease.

Methods: Using the ELISA method (DLD-Diagnostika-GMBH for ADMA and BioSystems S.A. Reagens & Instruments Costa Brava 30, Barselona, Spain), the sera of 70 subjects have been examined (35 RA not-treated, 35 healthy control group).

Results: Of 35 patients with RA, presence of ADMA was detected in 13 patients (37.14%); RF was present in 17 patients (48.57%), while anti-CCP$_2$ antibodies were present in 23 patients (65.71%). Of 18 patients who were RF negative, ADMA was detected in 9 patients (50%), while 11 patients (31.43%) were anti-CCP$_2$ positive. In 17 RF positive patients with RA, ADMA was detected in 4 patients, while 12 patients (34.28%) were anti-CCP$_2$ and RF positive. In the healthy control group 8 patients (22.85%) showed ADMA positivity. There was moderate correlation between ADMA and anti-CCP$_2$ antibodies in the group of patients with RA (r=0.34).

Conclusion: There was an association between ADMA and anti-CCP$_2$ antibodies of the second generation in patients with RA.

Keywords

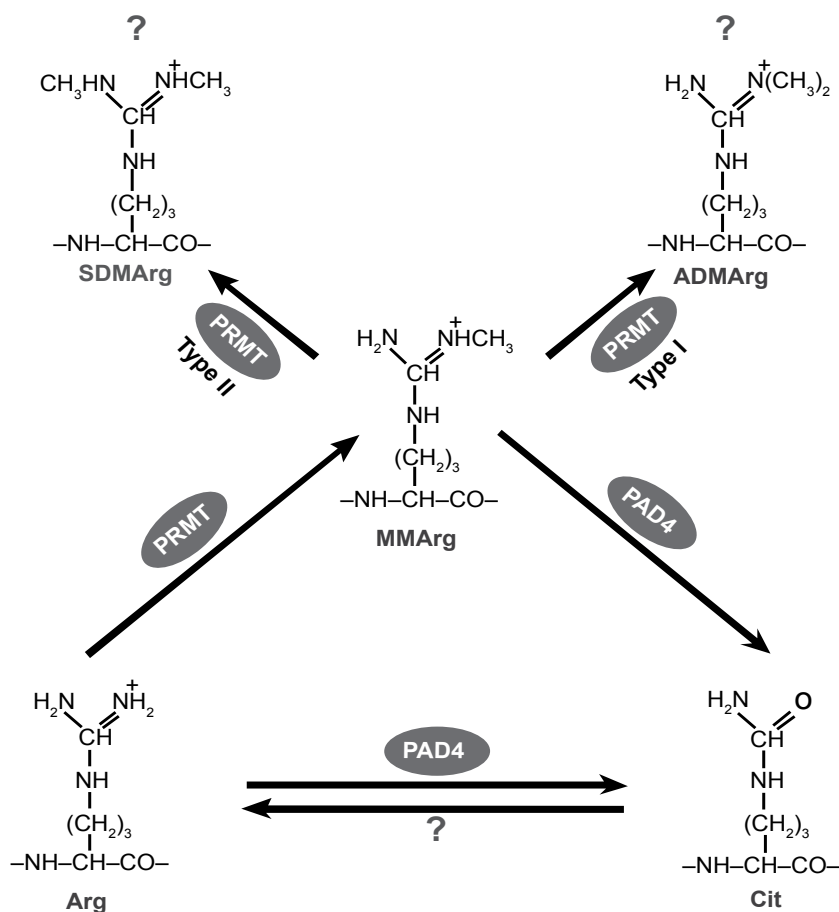Asymmetric dimethylarginine (ADMA), Rheumatoid arthritis, Rheumatoid factor

Introduction

The association between arginine and citrulline, i.e. between asymmetric dimethylarginine (ADMA) as dimethyl derivative of the amino acid L-arginine and anti-cyclic citrullinated peptide antibodies (Anti-CCP$_2$) of the second generation in patients with Rheumatoid Arthritis (RA) is the isoform of the enzyme Peptidylarginine Deiminase (PAD). Enzymatic deimination or protein citrullination is a process catalyzed by PAD enzymes which take part in the pathogenesis of RA. [1,2] In humans five PAD isoforms (PAD$_{1-4}$ and PAD$_6$) are present. The greatest attention is paid to PAD$_2$ and PAD$_4$, as potential candidates that could play role in the process of citrullination of autoantigens in RA. Both enzymatic forms are present in the rheumatoid synovium and fluid. [3-5] The polymorphism of PAD$_4$ is genetically associated with RA [6]. There is a difference in the tissue and cellular distribution of these two forms: PAD$_2$ is ubiquitously distributed in tissue (on the cytoplasmic level), while PAD$_4$ is more dominant in the hematopoietic cells (on nuclear level) [7-11]. In human neutrophils three different PAD isoforms (PAD$_{2-4}$) are expressed. PAD targets are different cells' substrates on cellular or sub-cellular level. In the process of intracellular protein citrullination, different auto-antibodies recognize these substrates as citrullinated substrates. But, this explanation is probably partial, because the protein citrullination can take place also extracellularly. Cellular targets, identified as potential citrullinated autoantibodies with extracellular distribution are: filaggrin, vimentin, β-actin, collagen type I and II, gamma enolase etc [12-19]. Extracellular citrullination of fibrinogen is probably derived by PAD from damaged cells [4,20], but, so far it is not known in which conditions it occurs. Citrullinated autoantibodies play key role in the immune answer in RA. Few unique characteristics of protein citrullination enrolled in the pathogenesis of RA are identified: 1. The cytoplasmic content has autoantigenic features and is a target in the process of citrullination; 2. Although there are great number of proteins citrullinated by the active neutrophils, anti- CCP$_2$ recognises only small number of these molecules; 3. Citrulline activity of each PAD isoforms is characterized by the unique substrate specificity, independently of

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Figure 1: Metabolic aspects of dimethyl derivates of the amino acid l-arginine and peptidylarginin deiminase (PAD).

their subcellular distribution. Only PAD_2 is capable of citrullination of native β/γ actine, while H_3 hystones are only citrullinated by PAD_4 isoform. PAD_4 isoform which is cytoplasmic enzyme similar to PAD_2 is not able to citrullinate actine and H_3 hystone; 4. Different reactive abilities of different sera in RA support the hypothesis that anti CCP_2 antibodies recognize the unique sequence [2,21]. The variations in the specificity of anti CCP_2 antibodies in different patients with RA are impressive. Probably all PAD isoforms share similar epitopes and their possible presence in the extracellular space indicate possible pass through the subcellular barriers.

Some Aspects of Dimethylarginine Derivatives of the Amino Acid L-Arginine

There are 2 stereoisomers of the L-arginine: symmetric (SDMA) and asymmetric dimethylarginine (ADMA). ADMA interferes with L-arginine in the production of nitric oxide (NO), which plays key role in the normal endothelial function. Namely, NO is synthesized in the endothelial cells with the enzyme - endothelial nitric oxide synthetase (NOS). The physiological substrate (precursor) for NOS in this enzymatic process is L-arginine, converted in NO and L-citrulline. The NOS is inhibited by ADMA. The plasma level of ADMA is elevated in RA.

ADMA is synthesized by protein methylation mostly in the cellular nucleus. The methylation is catalyzed by the group of enzymes called protein arginine N-methyl tranferases (PRMTs type I and II). Both PRMT subtypes have the ability to methylate monomethyl arginine (MMA); type 1 asymmetrically dimethylates arginine and creates ADMA, while type 2 catalyzes symmetrical dimethylation of arginine and creates SDMA (Figure 1).

Aim

The aim of this study is to determine the association between

ADMA and anti-cyclic citrullinated peptide antibodies (Anti-CCP_2) of the second generation in patients with Rheumatoid Arthritis (RA) and to explain their connection in the process of citrullination of autoantigens in RA.

Material and methods

In the patients examined for this study, the diagnosis of the disease was established on the basis of revised diagnostic criteria for the classification of RA, suggested in 1987 by the American Association for Rheumatism (ARA) [22]. In order for a patient to be diagnosed with rheumatoid arthritis, patients must fulfill at least four out of seven criteria. Criteria from one to four are present for at least six month. In order to be included in the study every patient should fulfill at least 4 of the predicted 7 criteria. Criteria of diagnosis are related to American Colleague of Rheumatology (ACR).

In the study are included 70 patients, 35 patients with RA (28 women and 7 men), as well as 35 patients as healthy control group (18 women and 17 men). The mean age is 56, 68 years (±6.79) (40-65 years) in the group with RA and 46.2 years (±12.49) (29-65 years) in the healthy control group. The median duration of the disease in months is 43.97 (±45,23) in the interval of 1-168 months. Three patients were previously treated with oral steroids, while nobody was treated with NSAIDs. The others denied the use of other drugs such as arginine or nitroglycerin before the entrance in the study.

Inclusion criteria

In the study are included patients with RA aged 18-65 years, previously non-treated with NSAIDs or DMARDs.

Exclusion criteria

In the study are not included patients with diseases or conditions that could directly or indirectly influence the results, such as: 1. Patients with

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previous history of spleen disease, thyroid disorders, hepatic damages, kidney, hematologic, neurologic and pulmonary disorders, autoimmune diseases, age <18 years; 2. Patients with Diabetes mellitus, acute infections, malignant diseases, febrile conditions ; 3. Patients with uric arthritis, urinary infections, SLE, mixed connective tissue disease, vasculitis; 4. Patients with previous history of blood trasfusions, as well as overweight patients; 5. Patients treated with basic drugs for RA are excluded from the study; 6. Also, patients with the presence of hypeglycemia and elevated level of degradation products (serum urea and creatinin, urine creatinin), smokers, patients with arterial hypertension, hematologic and enzymatic disorders detected in 0 point, are excluded from the study; 7. Patients treated with cardiologic and hypolipidemic drugs, oral contraceptive drugs (estrogens, progesterone), vitamin E, nitroclycerin, arginine or analogues, and patients who received inhalational anesthetics one month before the start of the study are also excluded.

All participants voluntarily participated in the study, so the ethic criteria for the preparation of the study were fulfilled.

clinical evaluation for disease activity

The clinical evaluation is made by the subspecialist in the field. Disease activity is evaluated using the DAS_{28} index (Disease Activity Score (DAS_{28}). [23] The index uses mathematical formula in order to obtain unique composite quantitative score which consists of palpable painful joints (maximal number – 28), swollen joints (maximal number – 28), Westergren's Erythrocyte Sedimentation Rate (ESR) and patient's global evaluation for the disease activity (0-100 mm Visual Analogue Scale -VAS) as morning stiffness (in minutes). The DAS_{28} index ranges from 0 to 10, and score bellow 3.2 qualifies the disease as low active.

Laboratory evaluation

For clinical evaluation of RA several variables were taken into account: Complete Blood Count (CBC), differential blood count, reactants of the acute phase such as C-reactive protein (CRP), Rheumatoid Factor (RF) and Erythrocyte Sedimentation rate (ESR), anti CCP_2 antibodies, Alkaline Phosphatase (AP), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Kreatinin Kinase (KK), Lactate dehydrogenase (LDH), serum urea and creatinin. The specimens are processed immediately (not frozen), respecting the rules of good laboratory practice.

Determination of the activity of the serum Asymmetric Dimethylarginine : ELISA method (DLD Diagnostika-GMBH)-Enzyme Immunoassay)

For quantitative determination of the endogen Asymmetric Dimethylarginine (ADMA) in plasma or serum

Principle: ADMA is connected in the solid phase of the micro titer plate. The ADMA standard, the specimens and positive control are pre-acylated in combination with rabbit anti-N-acyl-ADMA, and are incubated for 15-20 hours on 28^0C. Later they are washed and the second antibody is added - anti rabbit IgG conjugated with peroxidase. They are incubated for 1 hour on room temperature. After washing, in order to get dying, tetramethylbenzidine is added as a substrate solution (TMB). After 20-30 minutes of the incubation it is stopped adding sulfuric acid. The absorbance is read on microtiter read on 450 nanometers. ADMA concentration is determined with the standard curve. Basically the amount of antibodies connected in the solid phase of ADMA is conversely proportional to the ADMA concentration in the specimens.

Reference range: ADMA in serum is 0.4-0.75 micromol/L.

CRP is determined by the agglutination test (Lateks CRP test) (BioSystems S.A. Reagens & Instruments Costa Brava 30, Barselona, Spain).

Reference range: <6 mg/L CRP in serum.

RF is determined by the agglutination test (Lateks CRP test) (BioSystems S.A. & Instruments Costa Brava 30, Barselona, Spain).

Reference range: <8 IU/ml in serum.

Anti CCP_2 antibodies are determined by the ELISA method (BioSystems S.A. Reagens & Instruments Costa Brava 30, Barselona, Spain).

Reference range: <20 IU/ml in serum.

Quantitative method for determination of the ESR is the Westergren method.

Reference range: 7-8mm for men, 11-16 for women.

Statistical analysis

To test the significance of differences between two arithmetic means (proportions), the Student's t-test is used in comparison with the mean values of the determined numerical parameters between two groups, as well as Wilcoxon-matched test for independent specimens. The sensitivity and the prediction for the positive and negative test of the examined markers are determined with the test for sensitivity and specificity. The P-value in the range 0.05 and 0.1 is considered statistically significant. The data processing is made with the statistical package - Statistica 7.0.

Table 1: Laboratory results in RA and control healthy group (M ± SD)

	Non-treated RA Group N° 35 (M ± SD)	RA[sero-] N° 18 (M ± SD)	RA[sero+] N° 17 (M ± SD)	Control healthy group N° 35 (M ± SD)
	Positive/ negative	Positive/ negative	Positive/ negative	Positive/ negative
ADMA + > 0.75 (micromol/L)	13/22 1,096 (± 0,68) (0,25-3,1)	9/9 1,12 (± 0,58) (0,32-2,1)	4/13 1,07 (± 0,79) (0,25-3,1)	8/27 1,00 (± 0,50) (0,26-1,91)
RF + 30 > IU/ml	17/18 346,15 (± 625,22) (0,00-1920)	0/18 0,00 (± 0,00) (0,00-0,00)	17/0 712,67 (± 743,72) (30-1920)	2/33 13,71 (± 38,73) (0,00-120)
CRP + 12 > mg/L	14/21 46,86 (± 79,19) (0,00-384)	3/15 8,66 (± 24,62) (0,00-96)	13/4 87,31 (± 96,44) (0,00-384)	4/31 5,48 (± 12,80) (0,00-48)
ESR + > 16	27/8 48,62 (± 39,81) (2,0-120)	13/5 43,94 (± 39,82) (2,0-120)	14/3 53,58 (± 40,39) (5,0-120)	4/31 9,42 (± 8,21) (2,0-44)
ACPA antibodies + > 1,26	23/12 1,71 (± 0,69) (0,92-3,0)	11/7 1,56 (± 0,59) (0,93-2,6)	12/5 1,87 (± 0,77) (0,92-3,0)	1/34 0,95 (± 0,10) (0,90-1,38)
DAS 28 + > 3,2	28/7 4,79 (± 1,56) (1,85-7,03)	13/5 4,56 (± 1,76) (1,85-7,03)	15/2 5,04 (± 1,33) (2,47-6,83)	0/35 0,00 (± 0,00) (0,00-0,00)
Morning stiffness + > 0 min	26/9 43,20 (± 65,13) (0-300)	14/4 57,50 (± 81,40) (0-300)	12/5 28,05 (± 38,72) (0-120)	0/35 0,00 (± 0,00) (0,00-0,00)

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Table 2: Some diagnostic performance of laboratory variables in rheumatoid arthritis

	Rheumatic factor RA untreated group Number of patients 35	Rheumatic factor RA group[sero-] Number of patients 18	Rheumatic factor RA group[sero+] Number of patients 17	C-reactive protein RA untreated group Number of patients 35	C-reactive protein RA group[sero-] Number of patients 18	C-reactive protein RA group[sero+] Number of patients 17	Sedimentation RA untreated group Number of patients 35	Sedimentation RA group [sero-] Number of patients 18	Sedimentation RA group[sero+] Number of patients 17
Sensitivity %	48,57	0	100	66,66	16,66	76,47	77,14	72,22	82,35
Specificity %	94,28	94,28	94,28	88,57	88,57	88,57	88,57	88,57	88,57
Predictive values for positive test %	89,47	0	89,47	77,77	42,85	76,47	87,09	76,47	77,77
Predictive values for negative test %	35,29	35,29	0	40,38	36,60	11,42	20,51	13,88	8,82
Accuracy %	71,42	62,26	96,15	64,28	64,15	84,61	82,85	83,01	86,53

	Asymmetric dimethyl arginine RA untreated group Number of patients 35	Asymmetric dimethyl arginine RA group [sero-] Number of patients 18	Asymmetric dimethyl arginine RA group[sero+] Number of patients 17	Disease activity score (DAS 28) RA unreated group Number of patients 35	Disease activity score (DAS 28) RA group [sero-] Number of patients 18	Disease activity score (DAS 28) RA group[sero+] Number of patients 17
Sensitivity %	74,28	77,77	70,58	80	72,22	88,23
Specificity %	100	100	100	100	100	100
Predictive values for positive test %	100	100	100	100	100	100
Predictive values for negative test %	20,45	10,25	12,5	16,16	12,5	5,40
Accuracy %	87,14	92,48	90,38	90	90,56	96,15

Results

Of the 35 patients with RA, 13 patients (37,14%) showed presence of ADMA. RF was present in 23 patients (65.71%). Of 18 RF negative patients, ADMA was detected in 9 patients (50%), while 11 patients (31,42%) were anti CCP_2 positive. In 17 RF positive patients ADMA was present in 4 patients; 12 patients (34,28%) were anti CCP_2 and RF positive. In the healthy control group 8 patients (22,85%) showed ADMA positivity. RF was present in 2 patients (5,71%), while 1 patient (2,85%) showed anti CCP_2 positivity. In 35 patients with RA, $DAS_{28}>3.2$ was present in 28 patients (80%). In 17 seropositive RF patients, $DAS_{28}>3.2$ was present in 15 patients (88,23%) (Tables 1,2).

There is no statistical significance using Wilcoxon-matched test between ADMA in RA and the healthy control group for p<0.05 (p=0.555). 2. There is statistical correlation using Wilcoxon-matched test between ADMA in RA and age, duration of disease in months, DAS_{28} index, RF and CRP, ESR, morning stiffness, in the same group for p<0.05: (ADMA vs age p=0.000; ADMA vs duration of disease in months p=0.000; ADMA vs DAS_{28} p=0.000; ADMA vs RF p=0,018; ADMA vs CRP p=0,040; ADMA vs ESR p=0,000). 3. Although the presence of anti CCP_2 positive patients in $DAS_{28}>3.2$ seropositive and seronegative patients is almost the same (10 vs 9 patients), the range of anti CCP_2 titre is higher in 10 RF seopositive patients with $DAS_{28}>3.2$ than in RF seronegative patients with $DAS_{28}>3.2$ (2.23 ± 0.61vs1.92 ± 0.45). There is no statistical correlation between these two groups (p=0.374) (Chart 1). 4. There is statistical correlation using Wilcoxon-matched test between anti CCP_2 in RA and the healthy control group for p<0.05 (p=0.000), as well as between anti CCP_2 in RA and DAS_{28}, RF, CRP, ESR and morning stiffness in the same group for p<0.05 (anti CCP_2 vs DAS_{28} p=0.000; anti CCP_2 vs RF p=0,018; anti CCP_2 vs CRP p=0,041; anti CCP_2 vs ESR p=0.000, anti CCP_2 vs morning stiffness p=0,000). There is moderate correlation between ADMA and anti CCP_2 antibodies in the group of patients with RA (r=0.34) (Chart 2).

Discussion

The ADMA activity in serum is normally low. It is elevated in endothelial dysfunction, because of the presence of the chronic inflammation in RA. Its presence is quantified by the degree of inflammation in RA which correlates with the disease activity. There is a strong correlation between ADMA and anti-CCP_2 antibodies from the second generation in early RA as a result of the disease activity and chronic inflammation [24-26].

Seropositivity influences the ADMA induction (present also in our case) - seropositive RF patients with $DAS_{28}>3.2$ have higher ADMA induction than seronegative RF patients with $DAS_{28}>3.2$.

The association between ADMA and anti CCP_2 antibodies in patients with RA is confirmed also by other authors. They confirm the hypothesis that ADMA accumulation is associated with elevated titer of anti CCP_2 antibodies. Their conclusion is that excessive ADMA accumulation is associated with elevated titer of anti CCP_2 antibodies in patients with RA which duration is less than 3 years [25].

The association between anti CCP_2 antibodies and RF IgM in patients with RA and their predictive value is shown by other authors [27]. They conclude that anti CCP_2 antibodies and RF IgM are associated with the impaired endothelial function, independently from other cardiovascular risk factors. These autoantibodies could be reflection of the early atherosclerotic conditions and can induce increased risk of cardiovascular diseases.

The process of citrullination is desribed by other authors, also [28]. They demonstrate that human PAD_4 regulates the histone arginine methylation through conversion of methyl arginine in citrulline, releasing methylamine. The targets of PAD_4 are multiple areas in H_3 and H_4 histones that are methylated by the co-activators $CARM_1$ (H_3Arg^{17}) and $PRMT_1$ (H_4Arg^3). The decrease of the histone arginine methylation is secondarily associated with the increase in the process of citrullination. They point out that PAD_4 is mediated by the genetic expression through the regulation of arginine methylation and histone citrullination.

Other authors have almost equal perception [29]. They emphasize that PAD enzymes catalyze the conversion of arginine residues

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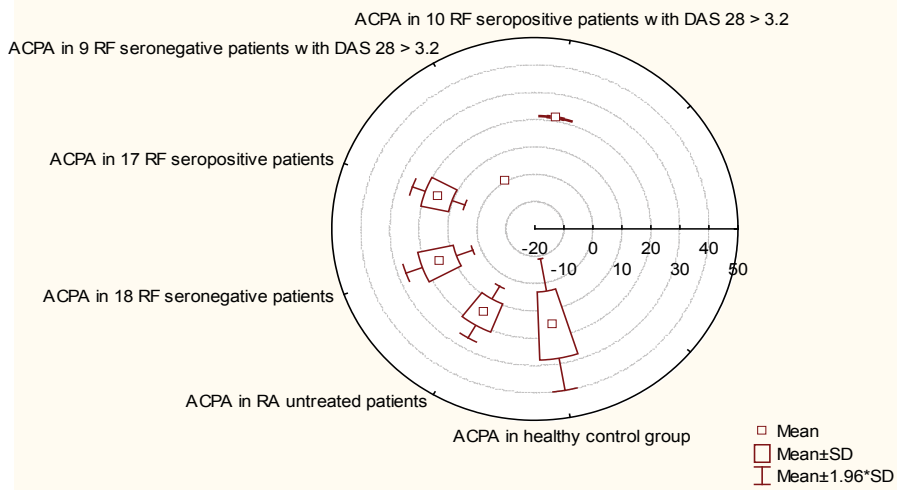
Chart 1: Distribution of ACPA antibodies in Rheumatoid arthritis in all groups.
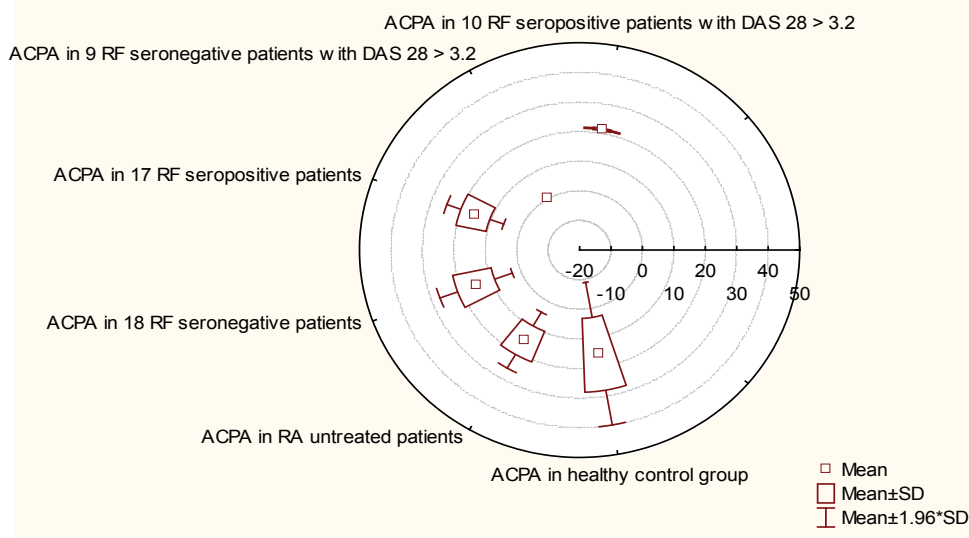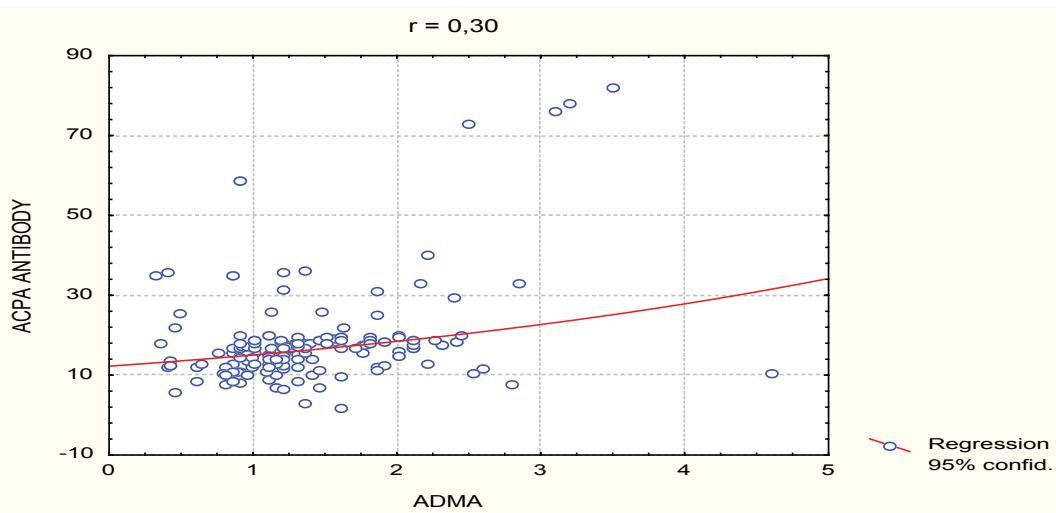
Chart 2: Correlation between ADMA and different parameter of RA. Pearson's coefficient of correlation (r) between the values of ADMA and ACPA antibodies in the group of patients with RA. There is a moderate correlation between ADMA and ACPA antibodies (r=0,30).

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in proteins with citrulline residues. The citrulline is not a standard amino acid. It is not incorporated in proteins during translation, but is generated post-translationally by PAD enzymes. In normal conditions only nuclear histones possess this amino acid.

Other authors also agree with the principle of auto-antigenicity in RA [30]. They relate auto-antigenic citrullination and different PAD enzymes in RA, emphasizing that PAD enzymes have intrinsic capacity for selection of protein targets. Such specificity could play role in auto-antigenic selection in RA. Other authors [31-33] conclude that citrullination of the proteins is enabled with the enzymatic conversion (by PAD enzyme) from proteins that contain arginine residues towards citrulline residues. They agree that PAD enzymatic activity fulfill the criteria as additive marker in monitoring disease progression, together with anti CCP_2 and RF. They emphasize that the PAD method for detection is rapid, sensitive and relatively cheap.

They suggest that citrullination and methylation of the arginine residues is a process of competition. It is theoretically reversible in regard of methylation of arginine residues through conversion of mono-methyl-peptydyl-arginine (MMA) in citrulline. But, contradictory results are shown in terms of the capacity of the PAD enzymatic activity towards citrullination of MMA. In regard of the reversibility of the arginine methylation, in general, the protein methylation is one-way process and the methylated arginine residues rest as an integral part of the proteins so far, until they are degraded in the process of proteolysis. The protein methylation, generally, is considered as an irreversible process, due to the lost of demethylating enzymes in the process of conversion.

PAD_4 is not able to citrullinate directly ADMA, but, it interferes in ADMA synthesis via citrullination of MMA as an intermediary product in ADMA synthesis.

Conclusion

ADMA is indirectly linked between autoantigenic citrullination and peptydyl-arginines' enzymatic isoforms in RA. Determination of ADMA together with other biological parameters contributes in the early diagnosis of RA.

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