UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
PhD SCHOOL

PhD THESIS

ABSTRACT

IMMUNOLOGICAL PROFILE OF PATIENTS WITH EARLY RHEUMATOID ARTHRITIS AND TREATMENT RESPONSE ASSESSMENT

PhD COORDINATOR:
Prof. Univ. Dr. PAULINA LUCIA CIUREA

PhD STUDENT:
Carmen Mitran (Sîrbuleț)

CRAIOVA
2016

TABLE OF CONTENTS

STATE OF KNOWLEDGE ..... pg. 3
AIM AND OBJECTIVES OF THE STUDY ..... pg. 3
MATERIAL AND METHODS ..... pg. 4
RESULTS AND DISCUSSIONS ..... pg. 5
CONCLUSIONS ..... pg. 9
SELECTIVE REFERENCES ..... pg. 10

STATE OF KNOWLEDGE

Rheumatoid arthritis (RA) is a chronic, autoimmune, inflammatory disease, characterized by progressive joint damage from early stages. A common objective for all rheumatologists is to establish an early diagnosis, which is not very often easy to be assessed.

Imagistic evaluation has a major role in diagnosing and monitoring these patients and all the methods should be complementary, in order to establish some cost efficient algorithms for a proper therapeutic management. Ultrasonography (US), a valuable method more and more approached in this pathology, supplies details regarding synovial inflammation, the start point for future joint damage.

We considered that determining and integrating in clinical and imagistic context some immunological markers, could improve real time evaluation of early RA patients and could optimize the treatment. Therefore, we choose to determine three serological markers, two of them already included in ACR/EULAR 2010 (American College of Rheumatology/European League Against Rheumatism) criteria, antibodies against cyclic citrullinated peptides (anti-CCP) and rheumatoid factor (FR), along with anti RA33 antibodies and to evaluate the patients using US, in odred to assess the therapeutic response.

AIM AND OBJECTIVES OF THE STUDY

We aimed to evaluate a chort of early RA patients, both by determing immunological markes and by using US, in order to assess therapeutic response. The personal contribution centered on assessing the correlations between immunological profile and the aspects achieved by using US.

The objectives were:

- ❑ Evaluation of the presence and the titre of anti -RA33 antibodies in a chohort of early RA patients.
- ❑ Determining the presence and the titre of anti -CCP antibodies.
- ❑ Determining the presence and the titre of RF.
- ❑ Measuring the inflammatory markers, both at the momment of diagnosis and after 12 months of treatment.
- ❑ Evaluation of disease activity using $DAS28_{(4v)}$, both at the momment of diagnosis and after 12 months of treatment.
- ❑ US evaluation, using gray-scale and power Doppler, both at the momment of diagnosis and after 12 months of treatment.
- ❑ Establishing the possible correlations between immunological profile and disease activity.

- ❑ Analysing the correlations between US assessment and immunological markers.
- ❑ Identifying the correlations between disease activity and US evaluation.

MATERIAL AND METHODS

We performed a longitudinal, prosepctive study, which included 29 subjects, diagnosed with RA, according to ACR/EULAR 2010 criteria [28], in Rheumatology clinic, Emergency County Hospital of Craiova, between March 2014-October 2015, followed for 12 months. Also, we had 21 controls, without any acute or chronic inflammatory conditions or history of any autoimmune diseases. All the patients signed an informed consent.

Inclusion criteria were:

- ❑ Diagnosis of RA, according to ACR/EULAR 2010;
- ❑ Age over $\geq$ 18 years;
- ❑ Duration of the disease under 12 months.

Exclusion criteria were:

- ❑ Pregnancy and lactation;
- ❑ Cancer;
- ❑ Diangnosis of other autoimmune diseases;
- ❑ Patients with severe infections;
- ❑ Patients that don't sign the informed consent.

Laboratory tests were performed according to the manufacturer's kit indications. In order to determine anti-RA33 antibodies we used a Human anti-RA33 Assay kit; venous blood samples were centrifuged at 9700 rotations/minute, for 15 minutes, and the serum obtained was stored at -80 ºC until analyze. Anti-CCP antibodies were determined using fluorescence immunoassay, RF by latex method, C reactive protein (CRP) by immunoturbidimetry, and erythrocyte sedimentation rate (ESR) by Westergreen.

Functional status was evaluated using Health Assessment Questionnaire (HAQ) and disease activity by calculating 28-Disease Activity Score (DAS), with 4 variables.

US was performed from the second to the fifth metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints and wrists (RC) joints, in dorsal side of both hands. Moreover, the second and fifth MCP joints were scanned also in lateral aspects.

The examiner used a MyLab 25 machine (Esaote SpA Genoa, Italy) with a 10-18 MHz frequency linear probe, according to EULAR guidelines [18] and noted synovitis both in grayscale (GS) and Power Doppler (PD). PD examinations were carried out using a Doppler

frequency of 8.0 MHz and a pulse repetition frequency of 750 Hz [19]. All examinations were performed by an expert sonographer, blinded for clinical and laboratory data, according to OMERACT-EULAR consent and OMERACT preliminary definitions

Statistical analysis was performed using GraphPad Prism 5.5. Results are presented as mean± SD and data were analyzed using t-test and One-way ANOVA for comparing groups, and Pearson/Spearman's coefficient for evaluating correlations. We considered a level of $p<0.05$ statistically significant.

RESULTS AND DISSCUSIONS

Initial evaluation

We included 29 patients, with a mean age of 41.72±9.38 years, most of them women (28).

Age (years)	41.72±9.38
Sex (women), n; %	28 (96.55%)
Disease duration (months)	6.72±2.55
ESR(mm/h)	48.76±37.27
CRP (mg/l)	12.37±1.85
anti-RA33(ng/ml)	0.139±0.138
RF (UI/ml)	86.65±105.7
RF positive (%)	62.08
anti-CCP (UI/ml)	254.0±531.3
anti-CCP positive	17 (58.62)
TJC	8.24±1.88
SJC	3.75±1.52
VASp	66.20±1.65
VASm	59.31±7.22
DAS28 $_{(4v)}$	4.61±0.76
HAQ	1.23±0.50

Assessing inflammatory markes revealed a mean value of 48.76±37.27 mm for ESR, 12.37±1.85 mg/l for C-reactive protein, and for fibrinogen 473.29 ±137.32 mg/dl.

	Mean	***SD***	***Min***	***Max***
ESR (mm/h)	48.76	37.27	15	110
CRP (mg/l)	12.37	1.85	5	54
Fibrinogen (mg/dl)	437.29	137.32	304	847

Immunological profile has an important role, both for diagnostic and for prognosis purpose. Determining the optimal antibody profile constitued the objects of several studies, with different results, depending on the design, geographical area, durations of the symptoms and age of the patients.

Our results showed a 58.62% percentage of seropositive patients, for anti-CCP antibodies, with a mean value of 254.0±531.3 UI/ml. Several studies have analyzed the diagnostic sensitivity for these antibodies, and a recent meta-analysis, which included over 18000 patients with RA, reported a percentage of 71.7 for anti-CCP antibodies. Moreover, in patients with early RA, the positive percentage was 61.6%

RF, a family of antibodies that constitute a diagnostic criteria, and also a prognostic marker, can be found present in about 60-80% of the cases with a long history and only to about 50% of early cases; our study revealed a percentage of 62.06 seropositive patients. Determining both anti-CCP antibodies and RF can increase the predictive diagnostic value.

Anti-RA33 antibodies, present in about 2/3 of the patients with early RA, have a variable sensitivity, between 6 and 58%, depending of the ethnic group, geographic area or severity of the disease, and a specificity up to 96%. Our results found a mean value of 0.139±0.138 ng/ml, statistically significant different of the value calculated for control group (p=0.0103), with 48.27% (14) positive patients. Therefore, we assessed a sensitivity of 40% and a specificity of 90%. A recent meta-analysis, that included all published data between January 2000 and September 2015, concluded that anti-RA33 antibodies are highly specific for RA, with a percentage up to 90%. Simliar results were reported by *Nell VPK et al*, in 2005, in a cohort of 102 patients with early RA and by *Al-Mughaleset al* in 2015. Of the 18 patients with positive RF, 4 had also positive titres of anti-RA33 antibodies. The presence of anti-RA33 antibodies in patiets with positive RF, can predict a less erosive disease.

	Mean	***sd***	***Min***	***Max***
RF (UI/ml)	86.65	105.7	8	365.87
anti-CCP (UI/ml)	254	531.3	7	2813
anti-RA33 (ng/ml)	0.139	0.138	0.078	0.833

Ultrasonography, more and more approached in daily practice, in order to assess early inflammatory changes, gives us the possibility of using it every time a patients comes for an evaluation. In our cohort, using GS, we found synovitis in all 29 subjects; PD examination revealed grade 1 for 20 (68.96%), grade 2 for 23 subjects (79.31%) and grade 3 for 6 (20.68%), with a mean PDUS score of 7.31±0.70.

Analysing the correlations between US changes and disease activity, we established mean number of 6.93±1.33 joints with active synovitis in patients with a high disease activity, compared to 5.21±1.85 for the ones with a moderate diseas activity, statistically significant (p=0.007). We also established a positive correlation between both GS and PD findings and disease activity. The study published by *Scire et al*, also undelined a significant correlation between these variables, as well as the study published in Arthritis&Rheum, în 2009, *by Bachaus et al*, *Watanabe et al* in 2012, in Clinical Rheumatology, *Naredo et al* in 2007, Arthritis&rRheum, etc.

	DAS 28(4v)		
	r	p	95% CI
GSUS	0.453	0.013	0.10-0.70
PDUS	0.427	0.020	0.07-0.68

Analysing the correlations between immunological profile and disease activity found a negative correlation for anti-RA33 antibodies, r=-0.3683, p=0.049; for anti-CCP antibodies we identified a positive correlation, r= 0.395, p=0.033.

	anti-CCP	**anti-RA33**	**RF**
r	0.395	-0.3683	0.313
p	0.033	0.049	0.096
IC 95%	0.022-0.671	-0.009 to 0.654	-0.069-0.618

12 months evaluation

Assessing disease activity we established a mean value of 2.82±0.33, with 17 patients (58.62%) having a low disease activity, 8 (27.58%) in remission and 3 (10.34%) a $DAS28_{(4v)}$, corresponding to a moderate disease activity. Remission rates were similar to the ones reported by *Scire et al*, in 2009 or de *Vasquez et al* in 2009. The baseline value of anti-RA33 weas signficant different between the three groups, p= 0.044. The finding of a lower anti-RA33 titre with a less severe evoultion was reported also by *Al-Mughales et al*, in 2015.

	Low DAS28	**Moderate DAS**	**Remission**
anti-RA33(ng/ml)	0.143±0.178	0.087±0.094	0.153±0.073
anti-CCP (UI/ml)	104.5±173.6	762.9±116	199.4±231.9
RF(UI/ml)	78.05±97.33	105.3±173.9	69.82±90.63

***Ultrasonographic evaluation*,** using GSUS, revealed synovitis for 25 of the 29 subjects, PDUS examination found active synovitis for 12, with a mean PDUS score of 2.24±3.02.

	GSUS -	**GSUS +**	**PDUS +**	**PDUS -**
Anti-RA33 (ng/ml)	0.1053±0.151	0.144±0.026	0.107±0.056	0.159±0.177
p	0.3772		0.031	
Anti-CCP (UI/ml)	139.4+212.7	703.3+1226	961+1179	125.6+191.7
p	0.490		0.0051	

Analysing the differences between the baseline anti-RA33 titre, depending on US evaluation, found a signifficant difference between patients with active synovitis and those without this finding. Moreover, we established a moderate correlation (r=-0.519, p=0.0039), between the two variables. The impact of thi simmunological marker on a less active disease was described also by *Harman H.* et al.

Both GSUS and PDUS are important in order to discriminate between the clinical remission with US subclinical inflammation and the real one, with no sign of US active synovitis, and can constitute the base of a prediction model that integrates several outcome measures. As it was shown by our results, patients with high titres of anti-RA33 antibodies had a milder evolution, with a low degree of US inflammation. These results led to the idea that remission can be predicted by a combination of clinical and immunological items and confirmed by US.

CONCLUSIONS

- RA is a chronic inflammatory disease, with a distructive evolution, that requires an early diagnosis and proper therapeutic management; an accurate diagnosis encloses to identify antibodies with high specificity and sensitivity for both diagnostic and prognostic purpose.
- anti-RA33 antibodies were found in about half of our cases, with a sensibility of 40% and a specificity of 90%.
- Ultrasonography (US), an imaging method with several advantages that made it lately indispensable in daily medical practice, has an unquestionable value in RA, and offered new opportunities by defining and outlining certain pathologic aspects regarding synovial inflammation, the characteristic hallmark of this disease
- Anti-RA33 antibodies are predictors of a less severe disease, most of our patients that had an increased baseline titre having a low disease activity or being in remission, after 12 months of treatment.
- After 12 months, analysing anti-RA33 titre, depending on US findings, we established statistically significant differences, between patients with/without active synovitis; a moderate, negative correlation between the two variables was established.
- For anti-CCP antibodies, we found different values, statistically significant, depending on the presence/absence of active synovitis.
- The current study shows that anti-RA33 antibodies might constitute an additional tool for diagnosing early RA patients, with a particular contribution in RF seronegative cases
- Determining additional antibodies, specific for a disease, with important impact on the evolution of the patients, and integrating the results with ultrasound findings, is not only improving the diagnostic, but can also help quantify the future articular damage and outcome of the patients.

SELECTIVE REFERNECES

1. Tobón GJ, Youinou P, Saraux A. The environment, geo-epidemiology, and autoimmune disease: Rheumatoid arthritis. Journal of Autoimmunity 2010; 35 (1) :10-4.
2. Harman H, Tekeoglu I, Kaban N. Factors influencing ultrasonographic remission in patients with rheumatoid arthritis. Rheumatol Int 2015; 35: 485-491.
3. Conrad K. Roggenbuck D. Reinhold D. Dorner Thomas. Profiling of rheumatoid arthritis associated autoantibodies. Autoimmunity Reviews 2010; 9: 431-435.
4. Aletaha D, Negoi T, Silman AJ, Funovits J, Felson DT, Bingham CO et al. 2010 Rheumatoid arthritis classification criteria: an Amercian College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 2010; 62: 2569-81.
5. Yang X, Wang M, Zhang X, Li X, Cai G, Xia Q, Wang L, Xin L, Xu S, Pan F. Diagnostic accuracy of anti-RA33 antibody for rheumatoid arthritis: systematic review and meta-analysis. Clin Exp Rheumatol. 2016 Apr 6. [Epub ahead of print]
6. Nell VPK, Machold KP, Stamn TA, Eberl G, Heinzl H, Uffman M, Smolen JS. Autoantibody profiling as early diagnostic and prognostic tool for rheumatoid arthritis. Ann Rheum Dis 2005; 64: 1731-1736.
7. Senolt L, Grassi W, Szodoray P. Laboratory biomarkers or imaging in the diagnostics of rheumatoid arthritis? BMC Medicine 2014; 12:49.
8. Vreju F, Ciurea M, Roşu A, Muşetescu A, Grecu D, Ciurea P. Power Doppler sonography, a non-invasive method of assessment of the synovial inflammation in patients with early rheumatoid arthritis. Rom J Morphol Embryol. 2011; 52(2):637-43.
9. Vreju FA, Chisalau BA, Parvanescu CD, Barbulescu A, Rogoveanu O, Firulescu S, Rosu A, Ciurea P. High Frequency Ultrasonography of the Hand in Rheumatoid Arthritis, Psoriatic Arthritis, Gout and Osteoarthritis Patients. Current Health Sciences Journal 2016; 42 (1): 35-39
10. B. Bruce, J.F. Fries. The Health Assessment Questionnaire (HAQ). Clinical and Experimental Rheumatology, 23 (2005), pp. S14–S18
11. Wells G, Becker J.C, Teng J, Dougados M, Schiff M, Smolen J,= et al. – Validation of the 28-joint Disease Activity Score (DAS28) and European League Against Rheumatism response criteria based on C-reactive protein against disease progression in patients with rheumatoid arthritis, and comparison with the DAS28 based on erythrocyte sedimentation rate. Ann Rheum Dis 2009; 68(6):954-960
12. Backhaus M, Burmester GR, Gerber T, Grassi W, Machold KP, Swen WA, Wakefield RJ, Manger B; Working Group for Musculoskeletal Ultrasound in the EULAR Standing Committee on International Clinical Studies including Therapeutic Trials. Guidelines for musculoskeletal ultrasound in rheumatology. Ann Rheum Dis 2001;60:641-9.

13. Torp-Pedersen ST, Terslev L. Settings and artefacts relevant in colour/power Doppler ultrasound in rheumatology. Ann Rheum Dis 2008; 67:143-9.
14. Al-Ani MM. Comparison of anti-filaggrin, anti-RA33 and anti-cyclic citrullinated peptide atibodies in the diagnosis of rheumatoid arthritis in Iraqi patinets. Iraqi J Comm Med 2012;337-340.
15. Lashkari M, Noori A, Hajiimanouchehri F, Oveisi S, Kazemifar M. Determination of specificity and sensitivity of anti-RA33 in diagnosis of early rheumatoid arthrtis. Glob J Health Sci.2014;6: 292-297.
16. Peluso G, Michelutti A, Bosello S, Gremese E, Tolusso B, Ferraccioli G. Clinical and ultrasonographic remission determines different chances of relapse in early and long standing rheumatoid arthritis. Ann Rheum Dis. 2011;70:172-5.