Study of serum level of ANA, CRP, CCP and RF in Iranian patients with rheumatoid arthritis compared with the healthy subjects

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ABSTRACT
Rheumatoid arthritis (RA) is an autoimmune disorder and the most common inflammatory arthritis affecting 0.5 to 1% of the general population worldwide. The cause of RA remains unknown. In active RA, bone turnover markers change in serum and urine before the appearance of pathologic signs in radiography. The aim of this study was to analyze the level of ANA, CRP, RF and CCP in patients with RA compared with the healthy subjects. A case-control study was designed. The study participants consisted of 50 patients with rheumatoid arthritis and 50 healthy controls. ELISA method was used to measure the levels of ANA, CRP, RF and CCP. Average level of ANA, CRP, RF and CCP in patients with rheumatoid arthritis was significantly higher than the control group with a mean concentration. This differentiation also was determined in patients with different sexes and ages. The mentioned factors can have some effects on disease diagnosis in the first level of disorder.

Keywords: Rheumatoid arthritis, ANA, CRP, RF, CCP

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INTRODUCTION
In the 1940s, the concept of autoimmunity in rheumatoid arthritis (RA) was proposed by Waaler, who threw light on the disturbances in the connective tissue metabolism involved in this disease [1]. RA is a chronic autoimmune disease that affects many systems and organs and has a frequency of half to 1% in the population. The disease is characterized by chronic inflammation of the synovial joints, and the chronic inflammation in RA causes erosions and deformities of the bones and articular cartilage [2]. Waaler demonstrated that the autoantibody RF is elevated in RA patients. In 1970, several years later, Steffen hypothesized that RA could be a collagen autoimmune disease [3]. Past clinical evidence has demonstrated the production of several autoantibodies, including RF and other anti-collagen antibodies such as CCP, ANA and CRP in synovial plasma cells in response to RA pathogenesis. These findings suggest local antigen activity in the immune response activation in synovial tissue [4]. Hitherto, and despite its nonspecificity, RF is still widely employed in the diagnostic work-up for RA [5]. RF are antibodies specific enough to be used as diagnostic and prognostic markers of RA. They often appear many years before the onset of clinical RA [6]. Recently, cyclic citrullinated peptide antibodies (anti-CCP) have come into use for the RA diagnosis. It has been reported that anti-CCP has quite a high specificity for RA about 98% together with a sensitivity similar to that for RF [7]. Although different studies reported variable results, it is known that anti-CCP antibodies are associated with active and erosive disease, like high RF titers [8]. However, there still was a need to explore other diagnostic markers with greater specificity for RA.
Substantial research has been conducted in this direction came up with newer interesting markers such as anti-MCV and, more recently, anti-CCP antibodies, which showed a satisfying specificity in immunodiagnosis of RA [9]. Anti-CCP antibodies were discovered while exploring the sera of RA patients for further autoantibodies distinct from RF and anti-MCV. The first citrullinated-binding autoantibodies in rheumatoid sera were discovered by Niehusand Mandema in 1964 [10]. These autoantibodies demonstrated the ability to bind to perinuclear granules in normal human buccal mucosal cells and were named antiperinuclear factor. Past studies also showed that these autoantibodies occur in 48% of RA patients and only in 1% of healthy controls[11]. Subsequent studies discovered that conversion of arginine to citrulline on peptides was essential for anti-keratin antibody and perinuclear factor binding. Hence, these autoantibodies were later called anti-citrullinated peptide antibodies [12]. A combination of anti-CCP and RF would increase the sensitivity of both tests. Most previous studies for evaluating the diagnostic value of anti-CCP have used RF as a gold standard. However, RF is known to have a low specificity and sensitivity [13]. C-reactive protein (CRP) was discovered by Tillett and Francis in 1930 [14]. They realized that serum from febrile patients formed a precipitate when mixed with a Streptococcus pneumoniae capsule component due to binding of CRP to phosphorylcholine, a major constituent of C-polysaccharide. CRP is a highly conserved protein belonging to the pentraxin family and is a key component of the acute-phase response to infection and inflammation. Several factors, such as age, sex, smoking and body mass index, have been shown to influence basal serum CRP levels in the absence of inflammatory stimuli [15]. CRP is encoded by the CRP gene, which is located on chromosome 1q23. CRP consists of two exons spanning 2.3 kb. CRP levels are under genetic influence [16] and several single-nucleotide polymorphisms (SNPs) in the CRP gene and their haplotypes have been associated with basal CRP concentrations and the magnitude of the acute-phase rise in CRP levels in active inflammation [17]. Antinuclear antibodies (ANA) are a specific class of autoantibodies that have the capability of binding and destroying certain structures within the nucleus of the cells [18]. ANA has been divided into specific subtypes based on the nuclear or cytoplasmic component they attack i.e. anti-DNA, antihistone etc. It is directly proportional to antibody concentration and expressed with a quantitative scale of values. Its evaluation is crucial as low titer is less significant than a high titer and may be seen even in healthy individuals. There are many studies which have attempted to determine the optimum screening dilution of sera for ANA testing[19].

The aim of this study was to characterize a population of patients with RA according to laboratory tests, including hemoglobin levels; evidence of inflammatory activity by C-reactive protein (CRP); presence of autoantibody rheumatoid factor (RF), cyclic citrullinated peptide (CCP) antibody, and antinuclear antibodies (ANA) in Iranian patients.

MATERIALS AND METHODS

Patients
This was a controlled clinical study. A total of 50 sera of Iranian RA patients were enrolled in this study. All patients were having established RA and meeting ACR classification criteria diagnosed by rheumatologist according to revised American College of Rheumatology (ACR) criteria for RA. A total of 50 healthy people were also studied. They were assigned as a control group. Inclusion criterion was RA which had been diagnosed by rheumatologist. An exclusion criterion was those suffering from connective tissue disorders. All patients and controls are from the same ethnic origin. The study was approved by the Ethics Committee at Islamic Azad University of Shahrekord Branch.

Detection of Autoantibodies
Measurement of RF (IgG, IgM and IgA) was performed at INOVA Diagnostics, Inc., San Diego, California, United States, using “Quanta LiteTM RF IgA ELISA”, “Quanta LiteTM RF IgG ELISA”, and “Quanta LiteTM RF IgM ELISA” assays (Inova Diagnostics, CA, USA), according to manufacturer protocol. We considered as cutoff points for positivity values higher than 15 IU/mL (IgM and IgA) and 20 UI/mL (FR IgG).

Antibodies to CCP were evaluated after generating a cyclic peptide from linear citrulline containing peptides, by substituting serine residues by cysteine. Anti-CCP were detected using ELISA as previously described with the linear peptides [20]. Control peptides had an unmodified arginine rather than citrulline. The cutoff level for positivity (OD, 0.3) was determined on the basis of generating 100% specificity for RA in previous assays using local normal controls.

ELISA for antibody binding to CRP
Antibody activity to mCRP was determined by an ELISA as described previously. CRP was obtained from Chemicon International Inc. (CA, USA). Polystyrene microtitre plates were coated with 100µl CRP
(2.5µg/ml in 0.15M Tris-HCl buffer, pH7.6) by overnight incubation at 4°C. After blocking with 200µl 1% BSA in Tris-HCl buffer for 30min, sera were diluted 1:200 and incubated overnight at 4°C in duplicate CRP-coated wells and duplicate bovine serum albumin (BSA)-coated wells. Bound antibody was detected with horseradish peroxidase (HRP)-conjugated goat antihuman IgG antibody (1: 2000). The peroxidase substrate OPD and H2O2 was used as the chromagen. Absorbancy at 490nm (A490) was measured using an automated spectrophotometer.

CRP was modified in 8M urea containing 10mM EDTA according to the method described previously. The preparation was then dialyzed against phosphate buffered saline (PBS) and insoluble material was removed by centrifugation. The capacity of urea/EDTA-modified CRP and native CRP to block antibody binding in sera to solid-phase CRP was measured by adding increasing amounts of native or modified CRP to sera with elevated anti-mCRP activity. The final serum concentration was 1:200, and the incubation time at room temperature was 1.5 h. The residual IgG antibody binding capacity to solid-bound CRP was determined by ELISA as described above.

**Statistical Analysis**

Statistical analysis was performed using SPSS statistical software version 20 (SPSS Inc., Chicago, IL, USA). The relation between RF, CRP, ANA and anti-CCP were analyzed with Chi square and T test. Values of p≤0.05 were considered significant.

**RESULTS**

The results of RF, CRP, ANA and CCP test in case and control groups was shown in table 1. The results of RF, ANA and CCP showed a significant differences between two mentioned groups (p value=0.001). In the other hand the result of CRP did not show a significant differences with p value=0.09.

<table>
<thead>
<tr>
<th>Methods</th>
<th>RF</th>
<th>CRP</th>
<th>ANA</th>
<th>CCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case</td>
<td>39.45±23.14</td>
<td>18.98±9.9</td>
<td>27.85±22.76</td>
<td>21.14±16.03</td>
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<tr>
<td>Control</td>
<td>23.78±21.77</td>
<td>11.23±5.84</td>
<td>20.47±19.31</td>
<td>7.4±5.51</td>
</tr>
<tr>
<td>Significance Level</td>
<td>0.001*</td>
<td>0.09[ns]</td>
<td>0.001*</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

The average results of RF, CRP, ANA and CCP test in case and control groups in different sexuality was shown in table 2. The average results of all tests showed that the control group has a significant differences in compare to case control in women (P≤0/05). This differences was not significant just for ANA test in men group (p value=0.45).

<table>
<thead>
<tr>
<th>Sexuality</th>
<th>Methods</th>
<th>RF</th>
<th>CRP</th>
<th>ANA</th>
<th>CCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td>Case</td>
<td>34.64±22.3</td>
<td>17.6±10.81</td>
<td>33.88±22.94</td>
<td>21.75±17.3</td>
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<tr>
<td></td>
<td>Control</td>
<td>23.88±23.03</td>
<td>12.12±6.85</td>
<td>19.44±19.06</td>
<td>6.3±6.07</td>
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<td></td>
<td>Significance Level</td>
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<td>0.02*</td>
<td>0.01*</td>
<td>0.008*</td>
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<tr>
<td>Men</td>
<td>Case</td>
<td>48.76±22.51</td>
<td>21.43±8.03</td>
<td>18.6±17.2</td>
<td>28.51±24.35</td>
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<tr>
<td></td>
<td>Control</td>
<td>23.59±19.97</td>
<td>9.8±3.37</td>
<td>22.23±20.16</td>
<td>9.34±6.97</td>
</tr>
<tr>
<td></td>
<td>Significance Level</td>
<td>0.002*</td>
<td>0.001*</td>
<td>0.45</td>
<td>0.003*</td>
</tr>
</tbody>
</table>

The average results of RF, CRP, ANA and CCP test in case and control groups with different age was shown in table 2. The average results of RF, ANA and CCP tests showed that the control group has a significant differences in compare to case control in the samples under 50 years of age. This differences was not significant for RF test in samples under 50 years of age (p value=0.14). Also, the average results of RF, CRP and CCP tests showed that the control group has a significant differences in compare to case control
in the samples over 50 years of age. This difference was not significant for ANA test in samples over 50 years of age (p value=0.45).

<table>
<thead>
<tr>
<th>Age</th>
<th>Methods Group</th>
<th>RF</th>
<th>CRP</th>
<th>ANA</th>
<th>CCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 50 years of age</td>
<td>Case</td>
<td>36.31±20.71</td>
<td>20.58±10.96</td>
<td>36.65±20.07</td>
<td>19.95±17.54</td>
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<tr>
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<td>Control</td>
<td>26.53±23.43</td>
<td>12.16±6.6</td>
<td>21.2±20.33</td>
<td>6.01±5.8</td>
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<tr>
<td></td>
<td>Significance Level</td>
<td>0.14ns</td>
<td>0.002*</td>
<td>0.012*</td>
<td>0.003*</td>
</tr>
<tr>
<td>Over 50 years of age</td>
<td>Case</td>
<td>41.99±25.03</td>
<td>17.72±9.16</td>
<td>22.64±20.74</td>
<td>25.26±24.04</td>
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<tr>
<td></td>
<td>Control</td>
<td>20.28±19.41</td>
<td>10.08±3.37</td>
<td>22.23±20.16</td>
<td>9.34±6.97</td>
</tr>
<tr>
<td></td>
<td>Significance Level</td>
<td>0.002*</td>
<td>0.001*</td>
<td>0.45</td>
<td>0.003*</td>
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</tbody>
</table>

**DISCUSSION**

Patients with RA follow a variable disease course with regard to outcome measures such as functional status or radiological assessment of joint damage. Early identification of patients with RA and, in particular, those likely to assume a more rapidly destructive form of disease, is important because of the possible benefit from early, aggressive intervention with disease modifying agents. This realization has prompted the investigation and measurement of numerous biologic "markers" in blood and joint fluids that may serve as indicators of prognosis and the response to therapy [21]. Although some biomarkers under consideration are accessible in routine practice, many are in the stage of experimental evaluation and require access to specialized technology and customized reagents. Rheumatoid factor may have some prognostic value with regard to disease manifestations and activity, and the severity of joint erosions. Seropositive RA (i.e., RA associated with a positive rheumatoid factor test) is often associated with more aggressive joint disease, and is more commonly complicated by extra-articular manifestations than seronegative RA [22]. RA is a chronic inflammatory arthritis specified by some antibodies. Although the most relevant antibody is RF, it has little sensitivity and specificity for diagnosis of RA. Other groups of antibodies that are produced against the citrullinated epitopes have been proved to be more specific for diagnosing RA [23]. RF is considered an early marker since its presence is linked with an increased risk of developing RA in people with mild arthritic symptoms. Rheumatoid factor became part of the classification criteria of RA almost 50 years ago. The main clinically useful biologic markers in patients with RA include rheumatoid factors, anti-CCP antibodies, ESR, and CRP. Measurement of anti-CCP antibodies also may be useful in the differential diagnosis of early polyarthritis. At present, the main clinically useful biologic markers in patients with RA are rheumatoid factors and antibodies to citrullinated peptides Anti-CCP Antibody test for both diagnosis and prediction of functional and radiographic outcomes, therefore testing for the combination of anti-CCP
antibodies and IgM RF may be better for excluding the diagnosis of RA than is achievable by testing for either antibody alone. The reported sensitivity of the RF test in RA has been as high as 90 percent. However, population-based studies, which include patients with mild disease, have found much lower rates of RF-positive RA (26 to 60 percent) [27]. In Finland the prevalence of RF-positive RA in adults was reported to be 0.7% [28]. The annual incidence has varied from 32 to 42 per 100,000 indifferent studies during the past two decades, [29] and was highest in eastern and lowest in western Finland [30]. In the absence of arthritis, the prevalence of positive and strongly positive RF reactions was 2.1% and 1.0%, respectively. A recent study from Finland suggested that daily coffee consumption was associated with an increased prevalence of "false positive" RF reactions and seemed to be a risk factor for RF-positive RA [31]. An Arabian study investigates the diagnostic reliability of anti-RA33 as an additional marker for RA compared to current immunodiagnostics markers including anti-CCP, RF, and CRP, among Saudi patients attending the rheumatology clinic at King Abdulaziz University's Tertiary Care Medical Centre. It was the first time that such a study has been conducted in the Saudi population, which should further provide valuable data regarding the prevalence of RA33 autoantigen in this population. Moreover, although the reliability of these fairly autoantibodies has been reported before, the data is still scarce, and this study therefore contributes to and builds on the already accumulated evidence [32]. In England the prevalence of a "false positive" RF reaction was higher in polluted areas than less polluted areas [33]. The prevalence of positive RF has been reported to be high in Pima Indians, [34] related to the high incidence of RA among the Pima [35] and declining in line with the temporal trends in RA [36]. The prevalence of RA is 0.5–1% among adults in Europe, but it seems to be much lower in some Asian and African populations [37]. Another study evaluated the genetic-environment-immune link in 515 Danish RA patients [38]. 309 had anti-CCP antibodies; of 456 tested, 262 had the shared epitope. The odds ratio (for RA) for those having both the shared epitope and having anti-CCP was 17.8. For the anti-CCP pos, shared epitope RA patients the odds ratio for those who smoked was 52.6 to 57.4 (vs 17.4 vs non-smokers); for those who consumed alcohol the odds ratio was 10.5 (vs 27 (for non-drinkers the odds-ratio was 50.1); the odds ratio for those who drank coffee was 27.4 to 53.3 (vs 13.0 for non-coffee drinkers); and the odds ratio for women who used OC was 44.6 (vs 32.3 for non-users). Furthermore the odds ratio for anti-CCP pos, shared epitope homozygous who were unmarried was 177 and those without a job was 243. The authors concluded that smoking among carriers of the shared epitope (either heterozygotes or homozygotes) accounted for 36 percent of all anti-CCP positive RA patients [39].

CONCLUSION
In conclusion, measurement of RF, ANA, CCP and CRP has good value as a screening test to diagnose or exclude rheumatic disease in either healthy populations or those with arthralgias. Our study is useful in estimating the probabilities of detecting RF, ANA and more specific anti-CCP and anti-CRP antibodies in serum samples referred to a rheumatology laboratory for detection and identification. According to the results of the study, average level of RA, CRP, RF and CCP in patients with rheumatoid arthritis was significantly higher than the control group with a mean concentration. This differentiation also was determined in patient with different sexual and ages. The mentioned factors can have some effects on disease diagnosis in the first level of disorder.

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