Correlation between DAS-28 and neopterin as a biochemical marker of immune system activation in early rheumatoid arthritis

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Abstract
Objective: The objective of this study was to evaluate neopterin plasma concentrations in patients with early Rheumatoid Arthritis (RA) and correlate them with disease activity. Methods: This is a 28-month prospective study carried out on 65 individuals. There were 27 patients with early RA and 38 healthy volunteers as control group. Enzyme immunoassay was used to measure concentrations of neopterin and anti-cyclic citrullinated peptide (CCP) antibodies. Rheumatoid factor (RF) and C-reactive protein (CRP) were measured turbidimetrically, and antinuclear antibodies (ANA) were detected by immunofluorescence. Patients with early RA disease activity were divided into 4 groups according to DAS-28 criteria. Neopterin concentrations in RA patients were compared to conventional RA diagnostic serological markers.

Results: Healthy volunteers had a mean neopterin concentration of 5.63 ± 0.38 [1.36–9.93] nmol. L−1. A statistically significant elevation of neopterin mean concentration was found on early RA patients: mean value of 8.92 ± 0.93 [3.94–28.3] nmol. L−1 (p < 0.001). Pearson product moment correlation suggests a correlation between neopterin concentrations and DAS-28 (r = 0.208, p = 0.065). The analysis of the mean values grouped according with the DAS-28 criteria showed a correspondence between these means, with a Pearson correlation coefficient r = 0.979, p = 0.021. CRP concentrations also showed a similar trend. Anti-CCP antibodies and RF revealed a positive relationship with RA activity. Such a correlation was not found with ANA results.

Conclusions: The elevation of plasma neopterin concentrations in early RA patients may indicate stimulation of immune response. Good correlation between neopterin concentrations and DAS-28 may facilitate assessing disease activity.

Keywords: Rheumatoid arthritis, immune activation, neopterin, DAS-28, rheumatoid factor, C-reactive protein

Introduction
Rheumatoid arthritis (RA) could be categorized as the paradigm of autoimmune diseases: it is a chronic inflammatory process that affects the synovia, cartilage, and bone. It affects 0.5–1% of the adult population worldwide, causing disabling morbidity [1]. Although the etiology of RA remains uncertain, recent studies of molecular and cellular mechanisms have clarified aspects associated with its pathogenesis. Autoimmunity manifested through the production of specific antibodies to the Fc fraction of Immunoglobulin G, known as rheumatoid factor (RF) or against cyclic citrullinated peptides, indicate the loss of tolerance mechanisms of T and B-lymphocytes.

The activation of the immune system leads to the production of cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and IL-8. These cytokines play a fundamental role in RA pathogenesis by regulating the expression of adhesion molecules and the increased migration and retention of leukocytes in inflamed tissue. It has been shown...
recently that soluble serum markers of immune activation and cytokine receptors such as soluble TNF receptor (sTNF-R), soluble IL-2 receptor (sIL-2R), and neopterin are elevated in patients with RA, correlating well with disease activity [2,3].

Neopterin, 2-amino-4-hydroxy-6-(D-erythro-1’, 2’, 3’-trihydroxypropyl)-pteridine, is a low-molecular compound derived from guanosine triphosphate (GTP) [2]. It is found in elevated concentrations in different body fluids during cellular immune response. Human monocytes/macrophages constitute the most important source of neopterin stimulated by interferon-γ (IFN-γ). There is a close relationship between the amount of neopterin produced by these cells and their capacity to produce reactive oxygen species (ROS). Therefore, neopterin concentrations may be considered as an indicator of oxidative stress caused by activation of the immune system [2,4].

Measurement of plasma neopterin concentrations has been proposed as a test for monitoring the activity of diseases such as hepatitis, HIV, measles, autoimmune diseases, as well as malignant tumors and allogeneic transplants [5]. Although plasma neopterin concentration measurements have been introduced in different countries for screening blood donations (Austria, Germany) [6,7], to the best of our knowledge, no studies of this sort have been carried out in Argentina substantiating its use as RA immunological marker.

Activation of the immune system plays an important role in a variety of infections, autoimmune diseases, malignancies, and transplants. Immune processes are also involved in neurological and cardiovascular diseases as well [5].

Appropriate and sensitive markers are able to detect immunologic changes to elucidate pathogenesis, detection of disease activity and therapeutic drug selection. Since 1967, when Sakurai and Goto isolated neopterin for the first time from human urine, numerous investigations recognized the biochemical, physiological, and pathological effects of neopterin, both in the course of infections and inflammatory diseases [8]. Measurement of neopterin in various human biological fluids has shown to contribute in monitoring cellular immune system activation in a simple and sensitive fashion [9].

The main objective of this study was to evaluate neopterin plasma concentrations in patients with early rheumatoid arthritis (RA) and correlate them with disease activity. Numerous studies have shown the importance of early diagnosis of RA [10], as it is well known that substantial irreversible erosive joint damage occurs within the first 2 years. Therefore sensitive markers and diagnostic criteria are needed to enable clinicians to diagnose the disease in early stages.

A prospective study was carried out in patients with early RA by a team of community hospital research physicians specialized in rheumatology and a researcher in the field of pterin and its derivatives. The investigators worked in close cooperation with the professional staff of a biochemical laboratory certified by ISO 9001–2008, accredited by the Argentine Biochemical Foundation (Fundación Bioquímica Argentina).

Materials and methods

Patients

The study was carried out from October 2007 to January 2010 and included 65 adult individuals (27 RA patients and 38 healthy volunteers). Written informed consent was obtained from all participating patients and controls. Patients were recruited from an outpatient clinic (Hospital Interzonal de Agudos Gral. José de San Martín) of the district of La Plata, Argentina. Patients enrolled included those with early RA (up to 2 years of symptoms onset) and fulfilled the American College of Rheumatology (1987) criteria. Patients excluded from the study were pregnant and puerperal women as well as patients with malignant diseases, patients with clinical manifestations of gastrointestinal, renal, hepatic, neurologic, hematologic, cardio respiratory or psychiatric diseases, metabolic disorders, and chronic or acute infections.

Plasma samples were obtained from the 38 healthy volunteers: 29 women and 9 men (mean age: 37.3 ± 12.8 yrs, median: 35 yrs) and 27 patients: 20 women and 7 men (mean age: 36 ± 12.6 yrs, median: 31 yrs). The healthy volunteer’s neopterin concentrations determined the limits of confidence for neopterin. Although increased neopterin concentrations have been demonstrated with increasing age, no significant relationship was found between neopterin concentrations and age in healthy volunteers and this group of patients (p = 0.627). RA patients had their baseline plasma samples taken at initial contact, at 6 and 12 months, respectively.

Estimation of the RA score was obtained for all patients using DAS-28 criteria recommended by the United Kingdom National Rheumatoid Arthritis Society (www.nrras.org.uk/DAS) [11]. The evaluation of this clinical parameter was calculated by the following equation: DAS 28 = 0.56 × (TJ)¹/² + 0.28 × (SJ)¹/² + 0.70 ln (ESR) + 0.014 GH (TJ: Number of tender joints from 28 joints; SJ: Number of swollen joints from 28 joints; GH: global health). Patients were divided into 4 groups according to DAS-28 criteria: Group DAS < 2.6: observed in patients with disease remission; group DAS from 2.6 to 3.2: characteristic of low disease activity; group DAS from 3.2 to 5.1: observed in patients who may have required changes in therapy; and, group DAS > 5.1: indicative of severe disease activity.
Additional clinical criteria considered for evaluating early RA, were VAS (Visual Analogue Scale) and HAQ (Health Assessment Questionnaire). At the time of their assessment, most patients were receiving treatment: steroids (prednisone, maximum dose: 10 mg per day), non-steroidal anti-inflammatory drugs (NSAIDs), methotrexate, hydroxychloroquine, leflu-nomide and sulphasalazine at conventional doses. Concentrations of neopterin did not differ between patients receiving different treatment regimens. Even though prednisone may influence neopterin concentration, there was no treatment-dependent difference in patients receiving steroid therapy ($p = 0.232$).

Measurements

Within the scope of routine blood examinations, fractions of serum and plasma with EDTA of patients were separated within 1 to 3 h from blood at 360 g for 15 min at room temperature. The collected samples were frozen at $-20^\circ$C until analysis. All steps involving serum neopterin concentration measurements were protected from direct light. Neopterin was determined by ELISA Genway Inc. (ref 40–371-25012 - Lots ENO 199 G; ENO 201 G; ENO 202 G). Neopterin concentrations were measured according to instructions provided by the manufacturer.

The criteria for acceptable performance were the target value plus or minus 2 standard deviations, as stated by the manufacturer and previous study [12]. Erythrocyte sedimentation rate (ESR) measurements were determined with the Westergren method. C-reactive protein (CRP) and rheumatoid factor (RF) were measured turbidimetrically (HITACHI Science Systems limited, Ibaraki, Japan) with a Hitachi 902 (Roche). Anti Cyclic Citrullinated Peptide antibodies (anti CCP) were measured with enzyme immunoassay (INOVA Inc., San Diego, CA, USA) by INOVA Inc., anti-nuclear antibodies (ANA) by immunofluorescence (IFA) in Hep-2000 cell line (Immunoconcept, Sacramento, CA, USA).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS), version 10.0. SPSS, Inc. (Chicago Illinois) was used for data analysis of this study. The verification of the confidence interval was calculated using the protocol C28-A3 from the Clinical and Laboratory Standard Institute (CLSI, 950 West Valley Road, Suite 2500 Wayne, PA 19087 USA). Fisher variance analysis and ANOVA were used to compare healthy volunteers and patients. P-values $< 0.05$ were considered to indicate statistical significance.

The non-parametric Mann–Whitney U-Test was employed for comparison of the 4 groups of patients described above. Pearson’s correlation analysis was applied to assess correlations. Standard deviation of the mean was evaluated as $\sigma/\sqrt{n}$, where $n$ is the number of patients under study in the different groups. To check the different mean values between DAS-28, neopterin concentrations and biochemical markers in the 4 groups, a standard linear correlation analysis was used.

Results

Intra-assay variability was evaluated with 2 different quality controls (QC); coefficient of variation obtained in QC normal and elevated level was: 8.0% and 6.1%, respectively. Inter-assay variability was 10.3% and 7.8%, respectively. Healthy volunteers’ neopterin concentrations determining the limits of confidence were within the range value recommended by the manufacturer ($<10$ nmol.L$^{-1}$). The lower and higher limits encountered were 1.36 (percentile 2.5) and 9.93 (percentile 97.5) nmol.L$^{-1}$, respectively. The mean neopterin concentrations detected in

![Figure 1. Correlation between neopterin results with the disease activity score (DAS-28) of the RA disease. The area depicted by the circle corresponds to low activity and lower concentration of neopterin in early RA patients.](image1.png)

![Figure 2. Correlation between DAS-28 (4 groups) and mean neopterin concentrations respectively. Regression line (—) and 95% confidence interval (---) are shown.](image2.png)
healthy volunteers was 5.62 ± 0.36 [1.36–9.93]. Mean neopterin concentrations in RA patients was 8.92 ± 0.93 [3.94–28.3] (p < 0.001). Neopterin concentrations in early RA patients were significantly higher than neopterin concentrations in healthy volunteers.

The neopterin concentrations obtained from early RA patients compared with the DAS-28 criteria are shown in Figure 1. Pearson’s analysis suggests a correlation between neopterin concentrations and DAS-28 (r = 0.208, p = 0.065).

The immune activation marker followed the increase of the clinical score. The analysis of the mean values grouped according with the DAS-28 criteria, showed a correlation between these means (r = 0.979, p = 0.021), as can be observed in Figure 2 and Table I.

This trend was more accentuated in patients with higher DAS-28. Nevertheless, the neopterin concentrations differences between early RA patients from group DAS-28 < 2.6 and from group DAS-28 > 5.1 were not statistically significant (p = 0.576). Mean values of neopterin, CRP, RF, and anti-CCP antibodies according with DAS-28 score groups described above are shown in Table I.

CRP results were analyzed using the DAS-28 criteria as well. A correlation was found between CRP and disease activity (r = 0.228, p = 0.044) (Figure 3b) as expected from previous studies [13,14]. The analysis of the mean values grouped according to the DAS-28 criteria, showed a correlation between these means (r = 0.8536, p = 0.146, Figure 3a). A statistically significant difference was observed between CRP concentrations from group DAS-28 score < 2.6 to group DAS-28 score > 5.1 (p = 0.001).

In this study, elevated concentrations of anti-CCP antibodies and RF were observed in the 4 groups of patients (Table I; Figure 4 and Figure 5). Concentrations of anti-CCP antibodies and RF tended to be higher in patients with DAS-28 > 5.1. ANA results were unrelated to disease activity (data not shown).

### Table I. Values (mean ± SD) of neopterin, CRP, DAS-28, anti-CCP and RF in the 4 patients’ groups.

<table>
<thead>
<tr>
<th>DAS-28</th>
<th>Neopterin nmol. L⁻¹</th>
<th>CRP mg.L⁻¹</th>
<th>a-CCP (units)</th>
<th>RF IU.mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.87 ± 0.13</td>
<td>7.56 ± 0.87</td>
<td>2.77 ± 0.65</td>
<td>152 ± 22</td>
<td>75.6 ± 17.6</td>
</tr>
<tr>
<td>2.6 &lt; DAS-28 &lt; 3.2 (n = 19)</td>
<td>8.83 ± 0.59</td>
<td>14.76 ± 6.22</td>
<td>161 ± 19</td>
<td>213 ± 58.6</td>
</tr>
<tr>
<td>3.2 &lt; DAS-28 &lt; 5.1 (n = 32)</td>
<td>8.50 ± 0.96</td>
<td>10.93 ± 2.83</td>
<td>158 ± 23</td>
<td>135.2 ± 40</td>
</tr>
<tr>
<td>DAS-28 &gt; 5.1 (n = 6)</td>
<td>11.25 ± 3.93</td>
<td>20 ± 9.15</td>
<td>229 ± 15</td>
<td>502.5 ± 108</td>
</tr>
</tbody>
</table>

### Discussion

Rheumatoid arthritis is a complex inflammatory disease of unknown cause. Early and sensitive markers are needed to evaluate the activation of the immune system and relate it to its pathogenesis as well as selecting the most suitable therapy for each patient [15]. In addition to contributing to RA clinical assessment, laboratory markers may help to diagnose and to measure disease activity and its severity. The most frequently used serum markers are RF, anti-

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CCP antibodies, ANA, and CRP. Although CRP parallels disease activity and is generally used to that end, measurement of neopterin concentrations was introduced in 1979 as a new indicator of immune activation. Our study aims at substantiating previous reports on the role of measuring neopterin concentrations to assess RA activity [2,5,16]. It is well known that upon stimulation with IFN-γ, monocytes and macrophages produce large amounts of neopterin and Reactive Oxygen Species (ROS) [5,17]. Cellular immune activation as well as the extent of oxidative stress may be estimated by measuring neopterin concentrations [2,18]. Consequently, the production of ROS during cellular immune activation might suggest their association with tissue damage occurring in RA [5,8,19].

Physicians evaluated disease activity using DAS-28 criteria. Neopterin and the other serum markers concentrations were analyzed in relationship to the clinical assessment. Early RA patients had higher mean levels of neopterin concentrations compared with healthy subjects ($p < 0.001$). A correlation was observed between neopterin concentrations and the clinical score DAS-28. Neopterin concentrations tended to be higher in patients with more disease activity. An incremental rise in neopterin concentrations was seen in patients with higher disease activity scores, despite there was no statistical significance between serum neopterin concentrations on patients in remission and those with high disease activity.

Elevated neopterin concentrations in patients with early RA as compared to controls indicate immune stimulation related with IFN-γ identified as a major participant in the inflammatory process [20]. Thus, the introduction of neopterin concentrations measurement in RA patients would allow monitoring of cellular immune response.

In reviewing RA serological markers, RF is one of the 7 classification criteria of the American College of Rheumatology for RA and one of the most widely used [21]. Its specificity increases considerably with higher values (RF > 50 IU/mL). Elevated concentrations of RF were found in about 90% of the cohorts of our study (Figure 4).

High concentrations of anti-CCP antibodies are the most specific serological marker for RA [22,23].

As with RF, the antibodies are present early in the course of the disease and may even precede its clinical onset. Anti-CCP antibodies showed no correlation with disease activity in the group of RA patients of our study. Nevertheless, concentrations of anti-CCP antibodies and RF tended to be higher in patients with more severe disease activity. Therefore, because of its high specificity, these antibodies are useful for diagnosis and prognosis [24,25]. CRP concentrations correlated well with disease activity. A statistically significant difference was observed between CRP concentration from DAS-28 < 2.6 to DAS-28 > 5.1 ($p < 0.006$), and a good correlation between different stages according to DAS-28 criteria and mean CRP levels ($r = 0.8536, p = 0.146$) [13]. No correlation was found between disease activity and ANA results.

No systematic study was carried out measuring disease activity with DAS-28 criteria and neopterin concentrations in patients with early RA. A recent study performed on long-standing RA patients was reported in the literature, analyzing the effectiveness of a biological therapy monitored by DAS-28 score and neopterin concentrations among other inflammation markers such as ESR and CRP. Biological treatments may impact the CRP levels out of proportion with clinical benefit [26]. Interestingly...
enough, a similar correlation of CRP concentration and disease activity was seen in our cohorts treated with conventional therapy (Table I).

There was an association observed between the mean value of the clinical score DAS-28 and the mean neopterin concentration measurements ($r = 0.979, p = 0.021$). This fact and the tendency of neopterin concentrations to be higher in patients with more disease activity may advocate the potential use of this biochemical marker in assessing disease activity at RA different stages. Our study results suggest that neopterin concentration measurement could be a worthy immune activation marker for RA. Further studies are needed to assess the relationship between neopterin concentrations and RA activity and pathogenesis.

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