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REPORT

# Autoantibodies for diagnostic and prognosis in rheumatoid arthritis: Cuban immunoassay with citrullinated fibrinogen peptide

✍Goitybell Martínez Téllez[1], Bárbara Tórres Rives[1], Vicky Sánchez Rodríguez[1], Maité Martiatu Hendrich[1], Cira Rodríguez Pelier[1], Hilda Elisa Garay Pérez[2]

[1] Laboratorio de Inmunología, Centro Nacional de Genética Médica. Avenida 31 esquina 146 No. 3102, Reparto Cubanacán, Playa, La Habana, Cuba
[2] Laboratorio de péptidos sintéticos, Centro de Ingeniería Genética y Biotecnología. Ave 31 No. 15802 e/ 158 y 190, Cubanacán, Playa, CP 11600, La Habana, Cuba
✍goity@infomed.sld.cu

**ABSTRACT**

Rheumatoid arthritis (RA) is a chronic and autoimmune inflammatory disease. The objective of this work was to develop Cuban immunoassays to determine antibodies and evaluate the usefulness of antibodies against a citrullinated fibrinogen peptide for diagnosis and therapeutic clinical assessment of patients with RA. A novel immunoassay to determine antibodies against a citrullinated fibrinogen peptide was designed by informatics prediction of B cell epitopes and synthetized by chemical synthesis. For evaluate the analytical and diagnostic performance a case-control study was conducted. Participants were 162 patients with RA, 112 patients with other diseases and 50 healthy individuals. Commercial rheumatoid factor antibodies, second generation citrullinated peptides, mutated citrullinated vimentin, carbamylated vimentin and antiqueratin were determined by commercial immunoassays. A longitudinal study was conducted with 60 patients with early RA. Antibodies, C-reactive protein, erythrocyte sedimentation rate, activity index and response to methotrexate at six months were determined. Diagnostic performance of the antibodies against the citrullinated fibrinogen peptide was superior to the most commonly used commercial immunoassays, with a lower cost per patient (5.52 USD) than the second generation antibodies (14.00 USD). Patients with these antibodies had a higher risk of a lower response to treatment ($p = 0.0481$). The determination of antibodies against a novel citrullinated fibrinogen peptide has lower cost and great utility for the diagnosis and therapeutic clinical evaluation in RA. This work granted the Annual Award of the National Academy of Sciences of Cuba for the year 2019.

*Keywords:* rheumatoid arthritis, antibodies against citrullinated peptides, fibrinogen, immunoassay

**RESUMEN**

**Autoanticuerpos para diagnóstico y pronóstico en artrtis reumatoide: inmunoensayo cubano con péptido citrulinado del fibrinógeno.** La artritis reumatoide (AR) es una enfermedad inflamatoria crónica y autoinmune. El objetivo de este trabajo fue desarrollar un inmunoensayo cubano para determinar anticuerpos contra un péptido del fibrinógeno citrulinado y evaluar su utilidad para el diagnóstico y valoración clínico terapéutica de pacientes con AR. Se diseñó un inmunoensayo novedoso para determinar anticuerpos contra un péptido del fibrinógeno citrulinado diseñado mediante predicción informática de epítopos de células B y se obtuvo mediante síntesis química. Para evaluar su desempeño analítico y diagnóstico se realizó un estudio de casos y controles. Participaron 162 pacientes con AR, 112 pacientes con otras enfermedades y 50 individuos sanos. Se determinaron mediante inmunoensayos comerciales los anticuerpos factor reumatoide, anti péptidos citrulinados de segunda generación, anti vimentina citrulinada mutada y anti vimentina carbamilada. Se realizó un estudio longitudinal con 60 pacientes con AR temprana. Se determinaron los anticuerpos, la proteína C reactiva, la velocidad de sedimentación globular, el índice de actividad y la respuesta al metotrexato a los seis meses. El desempeño diagnóstico de los anticuerpos contra el péptido del fibrinógeno citrulinado fue superior a los inmunoensayos comerciales más utilizados, con un costo por paciente inferior (5.52 USD) a los anticuerpos de segunda generación (14.00 USD). Los pacientes con estos anticuerpos, tuvieron mayor riesgo para baja respuesta al tratamiento ($p = 0.0481$). La determinación de anticuerpos contra un péptido novedoso del fibrinógeno citrulinado tiene menor costo y gran utilidad para el diagnóstico y evaluación clínico terapéutica en la AR. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2019.

*Palabras clave:* artritis reumatoide, anticuerpos contra péptidos citrulinados, fibrinógeno, inmunoensayo

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## Introduction

Rheumatoid arthritis is a disease characterized by the chronic inflammation and the presence of autoantibodies [1-3]. Despite primary factors triggering RA remain unraveled, there are evidences on synovial inflammation caused by complex environmental and genetic interactions [4-6].

RA was commonly diagnosed following the recommendations by the American College of Rheumatology as on 1988, establishing six clinical parameters and rheumatoid factor (RF) antibodies as the only serological criterion [7] for RA positivity. Due to the relation of citrullinated proteins with the RA patho-

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genicity mechanisms [1, 5, 8], antibodies recognizing these protein antigens were added. In fact, the new criteria established in 2010 by the American College of Rheumatology (ACR) and the European Union League Against Rheumatism (EULAR) included it for RA diagnosis [9].

Several studies have demonstrated that those antibodies recognizing citrullinated proteins appear at the early stages of the disease, being of high prognostic value and specificity. They allow differentiating RA patients among patients suffering from other arthropaties and are predictive of joint erosion [9-11]. The first antibodies described against citrullinated peptides were the perinuclear factor and anti-keratin antibodies (AKA) [12]. Among the most common assays for determining antibodies against citrullinated peptides (CPs) there are the tests for second generation CPs (anti-CCP2), which are highly specific at early disease stages [12].

Recent studies have detected citrullinated proteins in the synovial tissue of swelled joints in RA patients, including vimentin, enolase, fibronectin, type II collagen, fibrinogen and fibrin [13]. These proteins are regarded as relevant candidates for triggering autoimmunity, and the determination of antibodies against them could increase the diagnostic value of current immunoassays against the disease [13, 14].

There has been also described in RA patients the presence of antibodies reacting with carbamylated-modified proteins, this being a post-translational modification related to the ethiopathogenic mechanisms of the disease [15]. So far, there were no studies in Latin America demonstrating the use of antibodies against carbamylated antigens for RA diagnostic purposes.

In this setting, there is an increasing need for developing specific tests to improve RA diagnosis at the early stages, and to differentiate it from other rheumatic diseases affecting the joints and the connective tissue. This is paramount especially in patient of poor prognosis, and for evaluating disease progression and response to treatment. Besides, adequate treatments followed by a periodical test of the inflammatory activity contribute to a better prognosis in RA [16].

Therefore, this work was aimed to develop a set of new immunoassays in Cuban labs to determine RA antibodies. Moreover, it was evaluated the usefulness of an immunoassay determining antibodies against a novel citrullinated fibrinogen peptide (anti-CFP), its value for diagnosis and clinical therapeutic evaluation of RA patients.

## Results and discussion

### Epitope prediction from fibrinogen and design and synthesis of a citrullinated peptide

The antigenic determinants of the α and β chains of fibrinogen were predicted starting from the tridimensional structure of the protein at the databank RSCB-PDB-101, by using the BepiPred 1.0 and Discotope 1.2 software. Two peptides were selected and covalently linked through a cysteine residue intermediate to epitopes in α chain (aa. sequence 210-220, citrullinated at position 216) and β chain (aa. sequence 45-55, citrullinated at 47). Both peptide subunits were citrullinated at two Arg residues, through positioning a terminal Cys residue and substituting the Cys-carboxyl terminus by a final carboxamide. The aa. sequence was KDLLPS-citrullin-DRQHCGH-citrullin-PLDKKREEC, spanning 1.9 % of the α chain (11/560 aas.) and 2.4 % of β chain (11/461 aas.) of fibrinogen.

Solid phase synthesis was done by the 9-fluorenylmethyloxycarbonyl/tert-butyl (Fmoc/tBu) method. Products were purified by preparative reverse phase-high performance liquid chromatography (RP-HPLC) with LabChrom (Merck Hitachi, Alemania) equipment. Purity was assessed by analytic RP-HPLC with AKTA 100 (GE Healthcare, EUA) equipment. Molecular mass was determined by electrospray ionization mass spectrometry (ESI-MS) with a Q-Tof (Micromass, England) spectrometer..

The designed peptide was obtained with a positive net charge (2+), an Arg/citrullin ratio of 1 and a molecular mass of 2916.46 ± 0.02 Da, coinciding with the theroretical molecular mass, at a purity of 98.3 %.

The RP-HPLC chromatogram for the F14P17 peptide and the ESI-MS spectrum are shown in figure 1.

### Immunoassay development

The IgG subtype anti-CFP antibodies were determined by an indirect enzyme-linked immunosorbent

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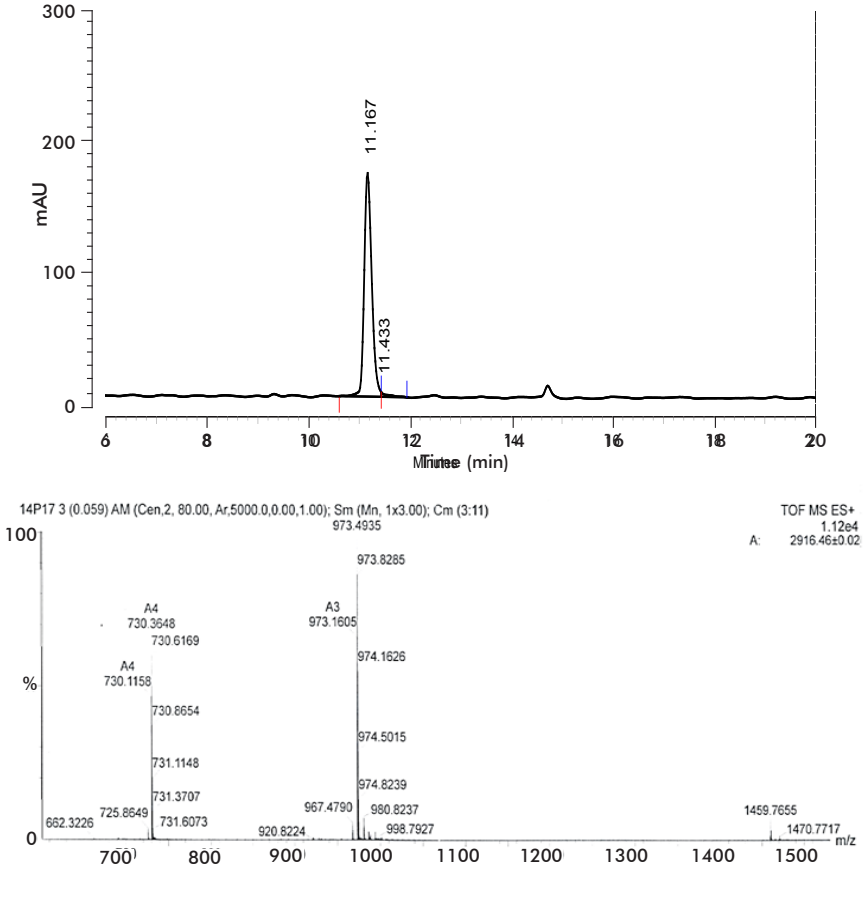


Figure 1. Analytical characterization of the F14P17 citrullinated fibrinogen peptide. A) RP-HPLC chromatogram of peptide F14P17. B) ESI-MS spectrum. A column RP-C18 (Vydac, 4.6 × 150 mm, 5 μm) was used. Gradient: 5-60 % of B disolution in 35 min. Disolution A: 0.1 % v/v trifluoroacetic acid in water. Disolution B: 0.05 % v/v of trifluoroacetic acid in acetonitrile. Detection was performed at λ 226nm. Theoretic monoisotopic molecular mass ($C_{120}H_{201}N_{43}O_{38}S_2$): 2916.46 Da.

assay (ELISA). Maximum absorption 96-wells plates (Maxisorp, Thermo scientific; Denmark) were sensitized with the fibrinogen peptide at 10 µg/mL in 0.15 M phosphate-buffered saline (PBS), pH 7.2. They were incubated in a humid chamber at 20-25 ºC for 16 h. Then plates were washed thrice with PBS plus 0.05 % Tween 20 in a microELISA equipment (SUMA, Cuba) at 200 µL per well, and further blocked by adding 2 % bovine serum albumin (BSA) (SIGMA, USA), for 1 h in a humid chamber at 20-25 ºC. Positive and negative controls were prepared from samples of RA positive patients and healthy individuals, respectively, diluted 1/100 in PBS plus 2 % BSA and 0.05 % Tween solution. Standard sera were added in the concentration range of 120-6.2 U/mL prepared from RA patients' sera, which concentration was previously assessed against the standard serum of the anti-CCP2 assay (IBL, Germany). After incubation at 20-25 ºC in humid chamber and as washing step, 100 µL the anti-IgG-peroxidase conjugate (Dako cytomation, Denmark) was added in every well at the recommended reagent dilution (1/6000) in PBS plus 0.05 % Tween 20. After another incubation and washing step, it was added 1 mg/mL orthophenylendiamine dichloride substrate (Sigma, USA) in 0.1 mol/L sodium citrate buffer, pH 5.5 plus 1 mg/mL hydrogen peroxide (Merck, Germany). The reaction was developed for 30 min at 20 ± 5°C, and stopped by adding 50 µL of 3 mol/L sulfuric acid (Merck, Germany) in water.

Results were measured by reading the plates at 492 nm in an ELISA plate reader PR-521 (SUMA, Cuba). A calibration curve with algorithmic adjustment was used to interpolate the values. The anti-CFP assay was developed with optimal conditions selected for the highest discrimination among optical densities of a positive control serum from RA positive patients and a negative control serum from healthy individuals, as recommended for immunoassay development [17].

### Analytical performance of the anti-CFP immunoassay

Analytical requirements of sensitivity, precision, accuracy and specificity were demonstrated according to CECMED regulations [18, 19], in the range 6.2-100 U/mL (cutoff value of 40 U/mL).

### Demographic and clinical characteristics of patients and its relation to antibodies

An analytical observational study was conducted of controls and clinical cases among 162 patients (81 with early RA and 81 with full established disease), older than 18 years, at the National Center for Rheumatology of Cuba, following ACR/EULAR criteria [9]. Patients with neoplasies or pregnant were excluded. Fifty healthy subjects were included, as well as 112 other subjects with other infectious, inflammatory and autoimmune diseases (26 patients with symptoms but not a definitive diagnosis of RA, 20 with mixed disease of the connective tissue, 20 with chronic viral hepatitis diseases, 18 with systemic lupus erythematosus, eight with sclerodemia, six with psoriasis arthritis, six with espondile arthropaties, six with autoimmune miositis and two with Sjögren's syndrome) at the Cuban National Center for Medical Genetics (CNGM). The research protocol was approved by the Ethics' Committee of CNGM, and the informed consent was provided by all the subjects enrolled.

It was determined the globular sedimentation speed (GSS) in 2 mL of blood anti-coagulated with 0.5 mL of sodium citrate (3.8 %) in 1 h. The reactive C protein was assessed by the qualitative technique of latex agglutination (Diagnostic Automation/Cortez Diagnostics, EUA). The RA' disease activity score (DAS) was calculated based on the count of 28 joints (DAS 28) with the GSS [20]. It was regarded as DAS 28 in remission (DAS 28 < 2.6), low activity DAS 28 ($3.2 \geq$ DAS 28 $\geq 2.6$), moderate ($5.1 \geq$ DAS 28 $> 3.2$) and elevated (DAS 28 > 5.1). The 20 %, 50 % and 70 % clinical improvement and the ACR's response to treatment criteria were also considered [21].

Anti-mutated citrullinated vimentin (anti-MCV), anti-carbamylated vimentin (anti-VIMCARB) and RF were determined by using the commercial reagents package from Orgentec, Germany, and anti-CCP2 with the assay package from IBL International, Germany. Patients included in the early RA had a median age of 48 years (range 39-53).

Upon diagnosis, 21 out of the 60 recently diagnosed RA patients showed a median duration of the disease of seven months (5-12). Established RA patients showed a median age of 51 (44-61), with a median duration of the disease of 5 years (2-10). Among female patients, the frequency was high both for early RA (76.5 %) and established RA (82.7%).

In the early RA, a positive correlation was found through the Spearman's test of anti-CFP antibodies to DAS 28 ($r = 0.2714$; $p=0.0142$) and RF ($r = 0.299$; 0.0066). In the established RA, a very high correlation was observed with prognostic value for anti-CCP2antibodies ($r = 0.5050$; $p = 0.0000$) and RF ($r = 0.4074$; $p = 0.0002$) with anti-CFP antibodies.

The presence of reactive protein C was associated to the presence of these antibodies in early RA patients ($\chi^2 = 5.8$; OR = 4.7; CI = 1.2-17.9; $p = 0.0163$) and in patients with established RA ($\chi^2 = 5.3$; OR = 2.9; CI = 1.2-7.4; $p = 0.0217$).

### Diagnostic performance of the anti-CFP immunoassay

Antibodies recognizing the vimentin and fibrinogen (anti-VIMCARB, anti-MCV, anti-CFP) showed higher diagnostic value, with a higher area under the curve (ROC) [22, 23]. Those joints's modified antigens, such as fibrinogen, have a significant capacity to develop auto-antibodies [13].

The anti-CFP antibodies showed a diagnostic performance higher than that of the most common commercial immunoassays (RF and anti-CCP2s) [23]. The diagnostic sensitivity and specificity, and the predictive positive and negative predictive values of the anti-CFP assay were, respectively: 72.2 (65.0-79.4); 88.9 (81.4-96.4); 92.9 (88.0-97.8); 61.5 (52.3-70.8).

Other researchers have stated that citrullinated fibrinogen could be important for RA, because antibodies against citrullinated peptides (anti-CPAs) could be of higher sensitivity for diagnosing the disease than other anti-CPAs and perform similarly to anti-CCP2s for diagnosis [24].

In 2014, Cornillet *et al.* evaluated the recognition for anti-CPAs of a peptide of the β chain of

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citrullinated fibrin (aa. 60-74, citrullinated at 60, 72 and 74) with a 71 % sensitivity and a 95 % specificity, similar to that obtained against anti-CCP2 (74 and 95 %, respectively) in the same population [25]. They also showed that those fibrin epitopes were recognized by antibodies recognizing the citrullinated fibrinogen. It was further studied the reactivity of fibrin epitopes against CPAs by combining a citrullinated peptide of the fibrin α chain (aa. 36-50) and another from the β chain (aa. 60-74) [25]. Ultimately, they reported a 47 % sensitivity and a 98 % diagnostic specificity [25].

Other studies showed a 98 % specificity and similar sensitivities (47 a 48 %) of the mix of a citrullinated peptide of α chain of fibrinogen (aas. 36-50) and one of β chain (60-74) [26].

It has been proposed that the sensitivity for anti-CCP2 antibodies is in the range 41-88 %, and 90-99 % for specificity [27, 28]. In fact, research in Latin America with anti-CCP2 antibodies varied 61.8-97.6 % for sensitivity and 52.5-97.0 % for specificity [4, 11, 14].

Previous studies evidenced that anti-MCV antibodies are a highly useful diagnostic tool, particularly at early RA and in anti-CCP2-negative patients [11].

Otherwise, some diagnostic value was found for anti-CCP2 antibodies, in agreement with previous results by Díaz-Tozcano *et al.* [11].

The sensitivity of anti-carbamylated vimentin (anti-VIMCARB) among Cuban patients with RA was higher than that evidenced by Ospelt *et al.* (50 %) with antibodies against carbamylated proteins [15].

Other studies have shown 60-80% sensitivity and 50-85 % specificity for RF IgM determinations in RA diagnosis [29]. On the other hand, AKAs have demonstrated a high specificity for RA diagnosis, despite a qualitative technique and the substrate being difficult to obtain [30].

## Clinical evolution, response to treatment with metotrexate and anti-CFP antibodies in early disease patients

Up to 60 patients with untreated early RA were reevaluated in a prospective longitudinal design six months after implementing the treatment with metotrexate, to measure the usefulness of anti-CFP antibodies to assess the clinical evolution and the therapeutic response. The 60 patients included were recently diagnosed and naive to treatment, 21 under treatment with metotrexate upon inclusion.

Untreated early RA patients were started treatment with metotrexate at 7.5 mg per kg of body weight weekly upon enrollment. Dosage was kept or increased as required up to a maximal dose of 25 mg weekly.

Prednisone was administered to all the patients at 10 mg daily during the first three months of treatment, following the rheumatologist recommendations.

Among established RA patients, 81.5 % were treated with metotrexate and 35.8 % were under treatment with other FAME as monotherapy or in combination with metotrexate as second therapeutic choice upon enrollment.

In fact, it has been recommended that RA should have to be treated for at least six months prior to determining the clinical response in patients, despite setting the three-month period as useful for clinical evaluations to establish if there was any improvement [31].

When re-evaluated after six months of treatment with metotrexate, a significant decrease was shown in the group of patients with anti-CFP antibody titers from a median value of 43,8 (29.7-69.1) U/mL to 33.0 (19.6-51.6) U/mL, as determined by the Wilcoxon test (p = 0.0242). Such a decrease coincided with a clinical improvement of patients.

It was evidenced a high to moderate DAS 28 decrease (p = 0.0000), from a median value of 5.2 (3.8-6.9) to 3.9 (2.8-5.5) after six months of treatment. Recently, Hensvold *et al.* observed a significant reduction in the ACPAs reacting against fibrinogen, vimentin and enolase, three months after treatment with metotrexate, together with a decrease in serum of the RANK ligand, which is a cytokine participating of joint damage [32].

Patients with early RA showing anti-CFP antibodies at the start of treatment showed a higher risk of being unresponsive or having a lower response (clinical improvement lower than or equal to 20 %) to metotrexate after six months of treatment than seronegative patients (Table 1).

Several studies have demonstrated that the presence of ACPAs at the start of treatment is indicative

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Table 1. Analysis of the influence of antibodies in the therapeutic response in Cuban patients with early RA

| Antibodies | Anti-CFP | | | Anti-MCV | | | Anti-CCP2 | | | RF IgM | | | RF IgA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | + | – | m(IQR) | + | – | m(IQR) | + | – | m(IQR) | + | – | m(IQR) | + | – | m(IQR) |
| Therapeutic response 20 % and NR (n = 44) | 40 | 4 | 43.7 (26.2-65.6) | 25 | 19 | 22.9 (13.7-83.5) | 24 | 20 | 42.6 (4.2-468.0) | 17 | 27 | 15.0 (2.0-41.9) | 28 | 19 | 26.9 (18.1-65.8) |
| Therapeutic response 50 and 70 % (n = 16) | 11 | 5 | 28.4 (15.6-45.3) | 11 | 5 | 32.2 (15.4-31.9) | 8 | 8 | 26.0 (3.8-54.7) | 6 | 10 | 10.0 (2.5-58.7) | 12 | 5 | 29.6 (17.3-46.9) |
| 20 % risk of response and NR | | | | | | | | | | | | | | | |
| Relative risk (95 % CI) | 1.8 (0.9-3.7) | | – | 0.9 (0.7-1.2) | | – | 1.1 (0.8-1.4) | | – | 1.1 (0.8-1.5) | | – | 0.9 (0.7-1.2) | | – |
| Chi-squared OR (95% CI); p | 4.5 (1.0-19.9); 0.0481* | | – | 0.6 (0.2-2.0); 0.4041* | | – | 1.2 (0.4-3.8); 0.7550 | | – | 1.5 (0.5-5.1); 0.4962 | | – | 0.6 (0.2-2.1); 0.3079 | | – |
| Mann Whitney p | – | | 0.0458* | – | | 0.0646 | – | | 0.0536 | – | | 0.7634 | – | | 0.8149 |

CFP: citrullinated fibrinogen peptide. CCP2: second generation citrullinated cyclic peptides. MCV: mutated citrullinated vimentin. Ig: immunoglobulin. IQR: inter-quartile range. RF: rheumatoid factor. n: number of patients. m: median. NR: non-responders. 95 % CI: 95 % confidence interval. OR: Odds ratio. * Statistically significant differences (p < 0.05). .

of a poorer prognosis and a lower response to metotrexate treatment [33, 34].

These results demonstrate the usefulness of anti-CFP antibodies for diagnosis and also as prognostic variable to measure the clinical evolution and the response to treatment. The presence of anti-CFP antibodies represents a higher risk of poorer clinical prognosis and a lower response to treatment. This relates to the role of citrullinated protein antigens such as fibrinogen for the RA ethiopathogenesis.

It has been observed that citrullinated fibrinogen is capable of activating macrophages through specific receptors, further contributing to the release of proinflammatory cytokines [35]. In this context, this antigen is presented to the immune system, leading to the break of tolerane as postulated for the RA immunopathogenesis [1, 2]. The sequential production of cytokines, antibodies and the formation of immunocomplexes involved in joint damage justify the fact that anti-CFP antibodiesare regarded as a factor indicating a bad clinical prognosis and a poor response to treatment.

The cost of the assay was lower for anti-PFC antibodies than the anti-CCP2 antibodies (5.52 vs. 14.00 USD, respectively).

In summary, the determination of antibodies against the citrullinated fibrinogen peptide was demonstrated as a novel and cheaper biomarker of good diagnostic performance and highly useful for prognostic purposes in Cuban RA patients.

## Relevance of the study

As a result of this research, a novel citrullinated fibrinogen peptide was discovered, able to improve current diagnostic systems against RA. It was found highly reactive against antibodies recognizing citrullinated proteins, these last generated throughout the disease. A novel immunoassay was developed and implemented for determining such antibodies. Its introduction supported the diagnosis and identification of patients displaying the severe form of the disease at the early stages and allowed the implementation of adequate treatments. It is also of high prognostic value to predict response to treatment and the activity of the disease.

All these avoided the acquisition of commercial immunoassays for testing national patients, proving cost-saving for RA diagnostic strategies at the Immunology Laboratory of the National Center for Medical Genetics of Cuba, with nationwide medical services through the National Network of Medical Genetics.

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## Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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