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A novel reactive epitope-based antigen targeted by serum autoantibodies in oligoarticular and polyarticular juvenile idiopathic arthritis and development of an electrochemical biosensor

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ABSTRACT

Currently, there are no specific markers for juvenile idiopathic arthritis (JIA) diagnosis, which is based on clinical symptoms and some blood tests for diseases' exclusion. Aiming to select new epitope-based antigens (mimotopes) that could recognize circulating autoantibodies in most JIA forms, we screened a phage displayed random peptide library against IgG antibodies purified from serum of JIA patients. ELISA assay was carried out to confirm immunoreactivity of selected peptides against sera IgG antibodies from JIA patients, healthy children and patients with other autoimmune diseases. The mimotope PRF + 1 fused to phage particles was able to efficiently discriminate JIA patients from controls, and for this reason was chosen to be chemically synthesized for validation in a larger sample size. The synthetic peptide was immobilized onto bioelectrodes' surface for antibody detection by electrochemical analyses through differential pulse voltammetry. The PRF + 1 synthetic peptide has efficiently discriminated JIA patients from control groups ($p < 0.0001$) with a very good accuracy (AUC > 0.84; sensitivity = 61%; specificity = 91%). The electrochemical platform proved to be fast, low cost and effective in detecting anti-PRF + 1 antibodies from JIA patients compared to healthy controls ($p = 0.0049$). Our study describes a novel and promising epitope-based biomarker for JIA diagnosis that can become a useful tool for screening tests, which was successfully incorporated onto an electrochemical biosensor and could be promptly used in field diagnostics.

1. Introduction

Juvenile idiopathic arthritis (JIA) is the most common inflammatory rheumatic disease of childhood (Ravelli and Martini, 2007). JIA is an autoimmune disease of unknown etiology that begins before 16 years of age and persists for at least 6 weeks. According to the International League Against Rheumatism classification, JIA can be classified in seven distinct subsets, being oligoarticular and polyarticular the most common (Petty et al., 2004). Up to now, JIA diagnosis is based on the combined evaluation of medical history of the patient, physical manifestation, radiographic imaging, and the detection of some serological markers (Petty et al., 2004; Martini

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and Lovell, 2010). Although JIA manifestations are influenced by genetic and environmental factors, studies focused on the identification of autoantigens that can drive the humoral response are of high interest to the clinical area. Currently, only the rheumatoid factor has been used for diagnosis of polyarticular forms of JIA (Duurland and Wedderburn, 2014); however, the lack of consistent biomarkers often delays diagnosis and makes the prediction of disease prognosis difficult. In this context, new biomarkers may be potentially used to guide decisions in the diagnosis and clinical management of JIA. Aiming to select novel peptides as potential serum biomarkers, we screened a phage displayed random peptide library against circulating IgG antibodies purified from JIA patients and submitted a selected peptide to further validation to check sensitivity, specificity and electrochemical properties.

The peptide selected against circulating autoantibodies that was able to discriminate JIA patients in the ELISA assay was successfully immobilized onto an electrochemical platform. This new sensor platform have reached a very good accuracy as a portable, low cost and fast diagnostic tool for autoantibodies detection in JIA.

2. Material and methods

2.1. Patients

For peptides selection, serum samples were obtained from Brazilian donors. A total of 40 JIA patients who fulfilled the diagnostic criteria for persistent oligoarticular ($n = 21$), extended oligoarticular ($n = 8$), polyarticular rheumatoid factor negative ($n = 5$) and polyarticular rheumatoid factor positive ($n = 6$), according to the International League of Associations for Rheumatology (Petty et al., 2004), 20 healthy children, and 17 patients with other autoimmune diseases were used. In order to increase specificity of the selected peptides, a subtractive selection step was performed using pooled serum from patients with rheumatic fever ($n = 5$), uveitis ($n = 3$), systemic lupus erythematous ($n = 4$) and Hashimoto's thyroiditis ($n = 5$). For synthetic peptide validation, well-characterized serum samples were obtained from Biobanco-IMM, Lisbon Academic Medical Center (Lisbon, Portugal). A total of 57 JIA patients who fulfilled the diagnostic criteria for persistent oligoarticular ($n = 21$), extended oligoarticular ($n = 7$), polyarticular rheumatoid factor negative ($n = 20$) and polyarticular rheumatoid factor positive ($n = 9$), 23 systemic lupus erythematous and 51 healthy children were enrolled in this stage. To minimize potential errors during the data analysis and improve the levels of sensitivity and specificity, samples of patients that presented inaccurate diagnosis at the time of sample collection were discarded.

Informed, written consent according to the declaration of Helsinki was obtained from parents of all study participants before any protocol procedure was carried out. This study was approved by the Research Ethics Committee from Federal University of Uberlândia (CEP-UFU; number 685/09), state of Minas Gerais, Brazil and by the Ethics Committee of the Centro Hospitalar Lisboa Norte, Hospital de Santa Maria, Portugal. Demographic and laboratory characteristics of the studied subjects are listed in Table 1.

2.2. Peptides selection by phage display

For peptides selection, a Ph.D.-C7C Phage Display (PD) Peptide Library Kit (New England Biolabs) was incubated with Immunoglobulins G (IgG) from the different groups coupled to magnetic beads according to the manufacturer's recommendations (Dynabeads® Protein G, Invitrogen). This library is based on a 7-mer random peptide combinatorial library fused to the pIII capsid of the M13 phage. For screening the PD library to select JIA mimotopes, we used a subtractive step against purified IgG from healthy children and patients with other autoimmune diseases, followed by a positive selection against purified IgG from JIA subtypes, which is strongly recommended to limit the recovery of target-unrelated peptides (Molek et al., 2011). The biopanning procedure was performed as described elsewhere with some modifications (Barbas et al., 2001). Briefly, 1×10^{11} phage particles of the PD library was incubated for 30 min at room temperature (RT) with beads coupled to IgG purified from serum of healthy children. After magnetic separation, the supernatant containing non-bound phages was transferred to a microtube containing beads coupled to IgG purified from serum of rheumatic fever patients, and incubated for 30 min at RT. The same procedure was performed for each autoimmune disease included in the control group. After subtractive step, the non-bound phages were collected and divided in four microtubes, each one containing beads coupled to IgG purified from serum of four JIA subtypes followed by incubation for 30 min at RT. Non-bound phages were removed after 10 washes with TBS-T 0.1% in the first round, and TBS-T 0.5% in the two subsequent rounds. The eluted phages were amplified in Escherichia coli ER2738 (New England Biolabs) between each round. The peptides selection was performed separately for each JIA subtype due to the fact that our initial goal was to identify biomarkers able to differentiate each subtype of the disease.

2.3. DNA sequencing of selected phage clones

After the third round of biopanning, phage clones were randomly picked up for DNA sequencing. Phages single-stranded DNA were isolated by iodide buffer extraction procedure (Instruction Manual Ph.D.-C7C Phage Display Peptide Library Kit) and analyzed with MegaBace 1000 Genetic Analyzer (Amersham Biosciences) automatic capillary sequencer using 200 ng of primer −96 gIII (5′-OH CCC TCA TAG TTA GCG TAA CG-3′; New England Biolabs). Nucleic acid sequences translation to the corresponding peptide sequences was performed using the ExPASy Translation tool (http://web.expasy.org/translate/) and the biochemical prediction of the PRF + 1 peptide primary structure was performed using the PepDraw tool (http://www.tulane.edu/~biochem/WW/PepDraw/index.html).

2.4. Phage-ELISA with the selected mimotopes

Phage-ELISA assay was first performed to test the reactivity and binding specificity of the selected mimotopes (phage clones) against pooled sera from JIA patients and healthy children (HC). Clones that presented significantly higher absorbance when compared to the HC were individually tested with sera from 40 patients with JIA, 5 with RF, 3 with UV, 4 with SLE, 5 patients with HT and 20 healthy children. Each pooled serum sample was tested against wild M13 phage (without displaying any peptide on its surface) as negative control. For data adjustment, the final optimal density (OD) was adjusted by subtracting the ratio of OD readings obtained by the tested mimotopes to the OD readings obtained by the wild-type M13 phage. All samples were tested in duplicate. Briefly, A ninety-six-well Maxisorp™ microtiter plate (NUNC, NY, USA) was coated with 1×10^{11} phages particles of each one of the selected phage clones diluted in 100 μl of carbonate buffer (0.1 M $NaHCO_3$, pH 8.6) and incubated overnight at 4 °C. The plate was blocked for 1 h at 37 °C with PBS-BSA 5% and then incubated for 1 h at 37 °C with pooled serum (1:250 in PBS-BSA 5%) from the three groups analyzed. The plate was washed 6 times with PBS-T 0.5% followed by incubation with HRP-conjugated goat anti-bovine IgG (Sigma–Aldrich) diluted (1:5000) in PBS-BSA 5% for 1 h at 37 °C. The plate was washed 6 times with PBS-T 0.5%, revealed with OPD SigmaFastTM (Sigma–Aldrich) and read at 492 nm.

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Table 1
Demographic and laboratory characteristics of the different groups included in the study.

Juvenile idiopathic arthritis = 97*									
	PO[a]	EO[b]	PR F−[c]	PRF+ [d]	Rhe F[e]	UV[f]	SLE[g]	HT[h]	HC[i]
n[j]	42 (21/42)**	15 (8/15)	25 (5/25)	15 (6/15)	5 (5/5)	3 (3/3)	23 (4/23)	5 (5/50	71 (20/51)
Gender (F/M)	31/11	10/5	21/4	11/4	2/3	2/1	13/10	0/5	46/25
Mean age ± [k]SD	11 ± 7	12.8 ± 8	14.1 ± 12	22.7 ± 14	9.6 ± 3.1	8 ± 4	14.3 ± 5.7	23.8 ± 2.3	12.9 ± 7.7
[l]RF positives	0	0	0	15	***	***	***	***	[m]N/A
Mean disease duration(years ± SD)	6.2 ± 5.6	9.1 ± 5.5	10 ± 8.1	16 ± 14.2	2.4 ± 0.3	1.3 ± 0.9	6.9 ± 3	4.7 ± 2.3	N/A
[n]CRP ± SD (mg/dL)	1.4 ± 1.8	1.1 ± 0.9	0.9 ± 1.2	2.2 ± 1.3	***	***	3.1 ± 1.1	***	N/A
[o]ESR ± SD (mm/h)	20.1 ± 18	19 ± 10.7	21 ± 14.8	20 ± 13	***	***	***	***	N/A

[a] PO = persistent oligoarticular.
[b] EO = extended oligoarticular.
[c] PRF− = polyarticular rheumatoid factor negative.
[d] PRF+ = polyarticular rheumatoid factor positive.
[e] Rhe F = rheumatic fever.
[f] UV = uveitis.
[g] SLE = systemic lupus erythematosus.
[h] HT = Hashimoto's thyroiditis.
[i] HC = healthy children; RA.
[j] *n* = number of subjects.
[k] SD = standard deviation.
[l] RF = rheumatoid factor.
[m] N/A = not applicable.
[n] CRP = C-reactive protein.
[o] ESR = erythrocyte sedimentation rate.
* Except for one patient, all JIA patients were tested in a previous publication (Araujo et al., 2015a).
** Total of samples used for peptides selection/total of samples used for synthetic peptide validation.
*** Missing data.

2.5. Peptide synthesis

The PFR + 1 peptide (SSWLPRG) that presented highest specificity in the phage-ELISA was chemically synthesized by GenScript USA Inc. (Piscataway, NJ, USA), and submitted for further analysis in a larger sample size. The peptide was constructed with 14 residues (ACSSWLPRGCGGGS) with amidation of the C-terminal region.

2.6. Synthetic peptide detection by ELISA

To determine the reactivity and binding specificity of the PRF + 1 synthetic peptide against circulating IgG in sera from JIA patients, patients with other autoimmune diseases and healthy children, ELISA assay was carried out. Briefly, a ninety-six-well Maxisorp microtiter plate (NUNC, NY, USA) was coated with the synthetic PRF + 1 peptide at 3 μg/ml diluted in carbonate buffer (0.1 M $NaHCO_3$, pH 8.6), and incubated overnight at 4 °C. Plates were blocked with 3% BSA in PBS at 37 °C for 1 h followed by incubation with serum diluted at 1:100 for 1 h at 37 °C under gentle agitation. After four washing steps with PBS-T 0.05%, HRP-conjugated rabbit anti-human IgG (Roche Applied Science) diluted at 1:5000 in blocking buffer was added followed by incubation for 1 h at 37 °C under gentle agitation. ELISA plate was washed three times with PBS-T 0.05%, the reaction was revealed with OPD SigmaFast™ (Sigma–Aldrich) and read at 492 nm. All samples were tested in duplicates. Each serum sample was tested alone (without PFR + 1 peptide) as negative control. The final OD was adjusted by subtracting the ratio of OD readings obtained for each serum sample to the ratio of OD readings obtained by the negative control. The optimum point of reaction for anti-PFR + 1 antibodies detection was determined using the receiver operating characteristic (ROC) curve, where a cut-off point was determined as the value of the parameter corresponding to the highest possible sensitivity without losing specificity. To estimate the positive predictive accuracy, the area under the curve (AUC) was also determined.

2.7. Electrochemical analysis

To test the immobilization of the PFR + 1 peptide onto gold electrodes and antibody detection, electrochemical analysis was carried out. Briefly, a 7 mm^2 ItalSens IS-Au gold screen-printed electrode was pretreated with cyclic voltammetry in sulfuric acid 0.5 mol L^1 and then a PFR + 1 peptide solution (1 μg mL^{-1}) was applied onto the electrode surface. The electrode was then incubated for 40 min at 8 °C and left to dry for 10 min at 37 °C. The surface was blocked with PBS-BSA 3% for 40 min at 4 °C, left to dry for 10 min at 37 °C, and separately incubated with serum samples from patients with JIA or healthy controls for 40 min at 4 °C. After incubation with serum, the surface was rinsed with 0.1 mol L^{-1} phosphate buffer (pH 7.4) for 6 s under gentle agitation. All measures were performed though differential pulse voltammetry: To detect the immobilization of the peptide, phosphate buffer 0.1 mol L^{-1} was used as the electrolyte, while 5 mmol L^{-1} potassium ferrocyanide ($K_4[Fe(CN)_6]$) solution containing 0.1 mol L^{-1} KCl was used to evaluate anti-PRF + 1 antibodies detection in serum samples.

2.8. Statistics

Unpaired *t*-test with Welch's correction was used to determine significant differences in reactivity between groups. All statistical analyses were performed using GraphPad Prism 5.0 software. *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Peptides selected by phage display

A total of 38 selected peptides presented different amino acid sequences (Table 2) and were tested for immunoreactivity evaluation by phage-ELISA (Fig. 1). Although most peptides were able to discriminate JIA patients from healthy children and patients with other inflammatory diseases, the PRF + 1 presented the highest specificity (Fig. 1E) and for this reason was chosen to be chemically

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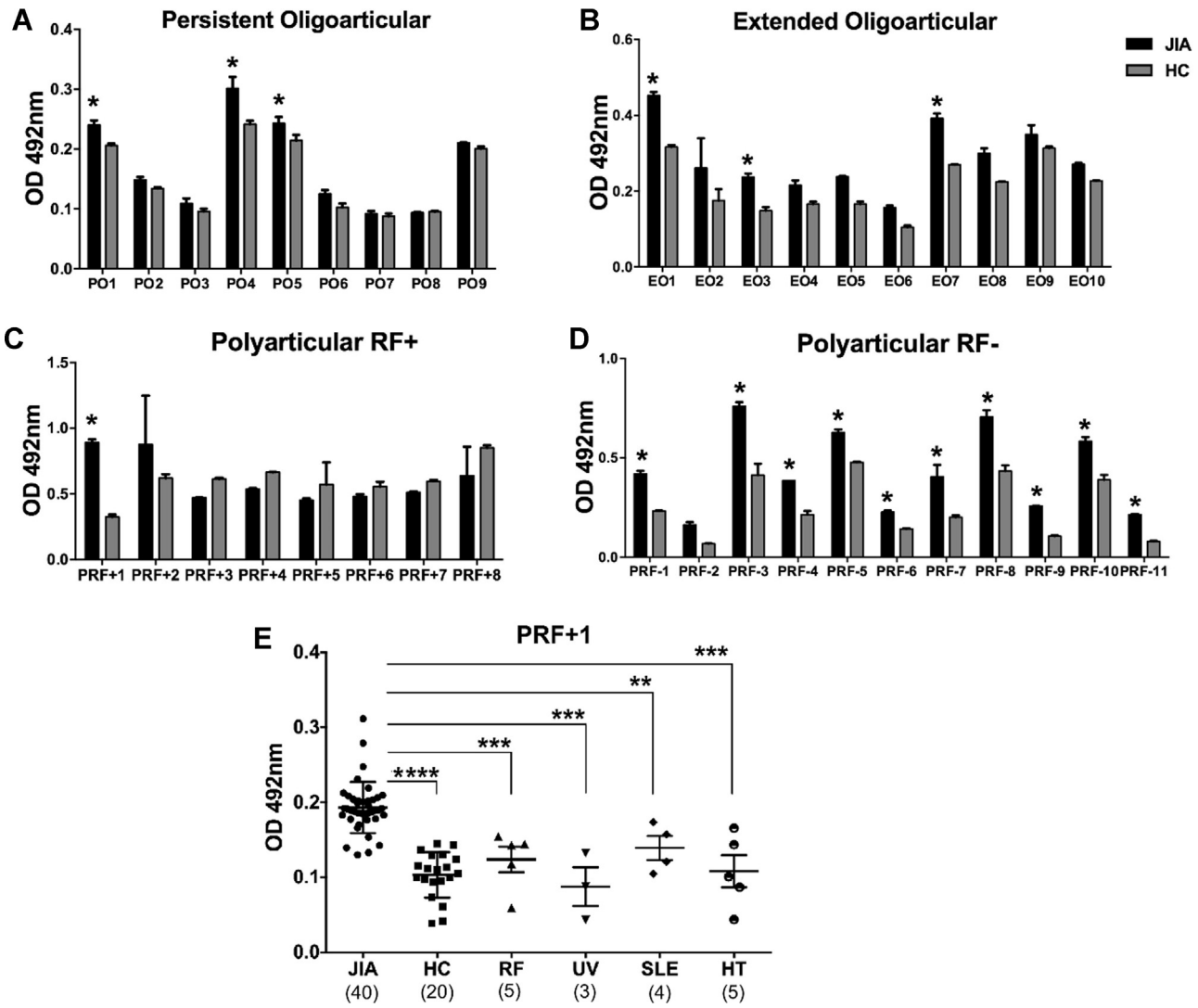

Fig. 1. Peptides selected by phage display and its immunoreactivities. Phage-ELISA assay was first performed to test the immunoreactivity of the selected peptides against pooled sera from different subtypes of JIA patients and healthy children (A–D). Peptides that presented absorbance significantly higher when compared to the healthy control (*) were tested against individual serum samples from JIA patients and control groups (E). The PRF + 1 peptide presented highest specificity in discriminating JIA patients from controls and was chosen to be chemically synthetized for further analysis and validation.

synthesized for further evaluation. The other peptides selected will be explored in forthcoming studies.

3.2. PRF + 1 synthetic peptide validation by ELISA

Based on the determined cut-off point, 57 of 97 (59%) JIA patients were positive for anti-PRF + 1 antibodies. The PRF + 1 synthetic peptide was able to efficiently discriminate JIA patients from healthy children ($p < 0.0001$) and other autoimmune diseases ($p < 0.0001$) (Fig. 2A). The ROC curve constructed was statistically highly significant (AUC = 0.8478; $p < 0.0001$) and based on the cut-off value determined, the PRF + 1 peptide presented sensitivity of 61% and specificity of 91% in diagnosing JIA (Fig. 2B).

3.3. Correlation between anti-PRF + 1 antibodies levels and studied variables

Anti-PRF + 1 antibodies detected in JIA patients were directly correlated with the number of swollen joints (Pearson $r = 0.4626$, $p = 0.0005$).

3.4. Anti-PRF + 1 antibodies detection by electrochemical analysis

The electrochemical analysis indicates the immobilization of the peptide onto the gold electrode surface. A biochemical structure prediction of the peptide showing the points of possible interaction with the gold electrode is presented in Fig. 3A. The differential pulse voltammogram in phosphate buffer of the bare gold electrode (Fig. 3B) was significantly different from the base line in the gold electrode with the immobilized peptide and between groups (Fig. 3C). The proposed bioelectrode was able to efficiently discriminate healthy controls from patients with JIA ($p = 0.0049$) through differential pulse voltammetry in potassium ferrocyanide solution (Fig. 3D).

4. Discussion

Different serum biomarkers have been reported for JIA diagnosis (Araujo et al., 2015a,b; Gibson et al., 2012; Kamiya et al., 2015), which include the detection of autoantigens–autoantibodies complexes and measurement of inflammatory markers in the peripheral blood, but none have been consistently used for

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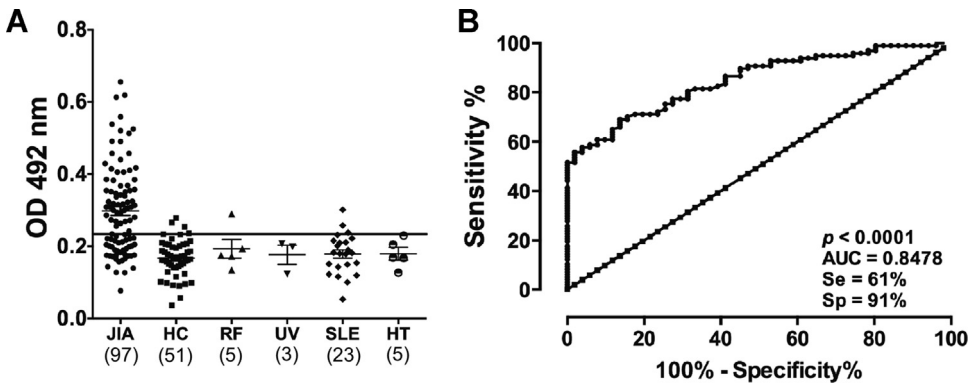

Fig. 2. (A) Anti-PFR + 1 antibodies detection by ELISA. (B) Anti-PFR + 1 antibodies detected in JIA patients and controls and the respective ROC curve.

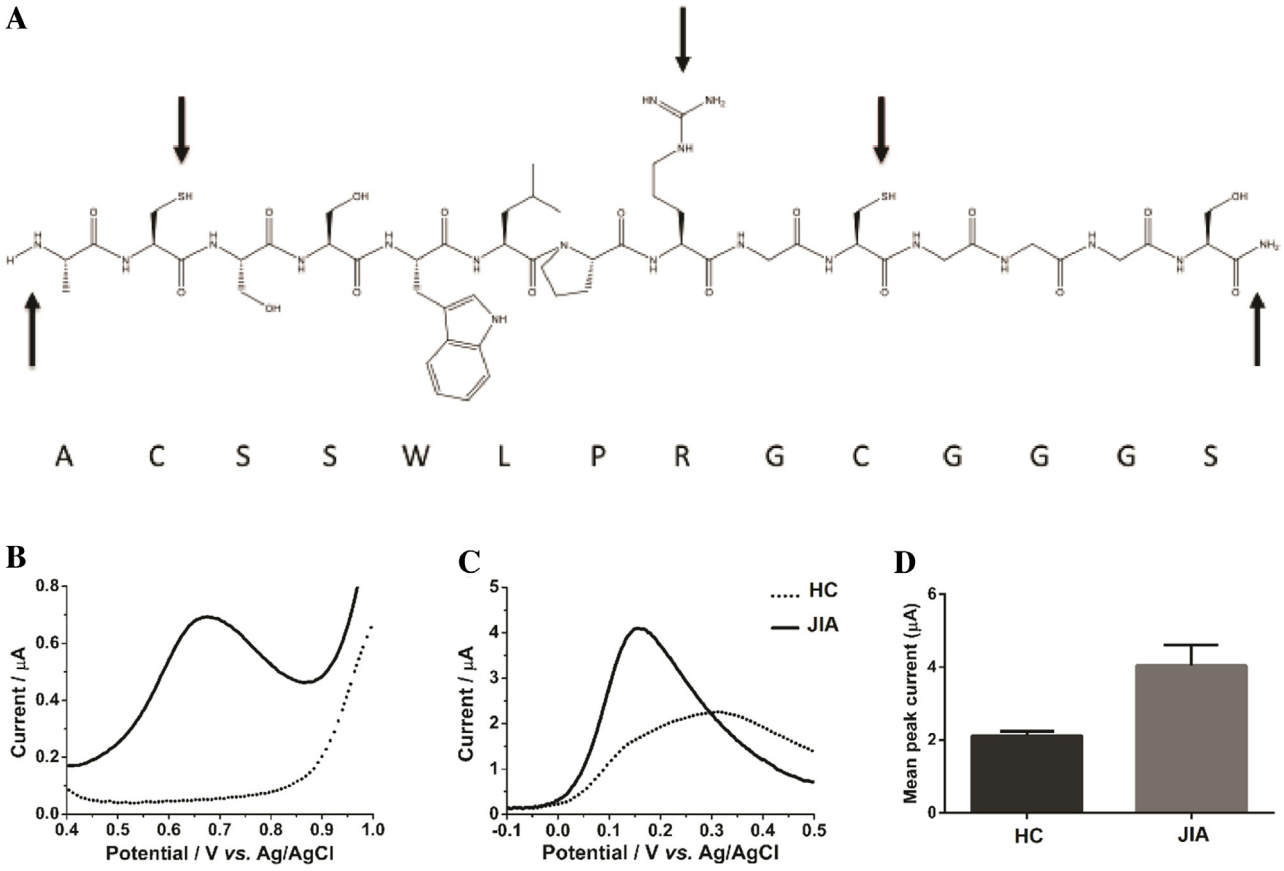

Fig. 3. Anti-PFR + 1 antibodies detection by the electrochemical platform. (A) Biochemical prediction of the PRF + 1 peptide primary structure. (B) Differential pulse voltammograms in phosphate buffer 0.1 mol L^{-1} of bare gold electrode (dashed line) and gold with immobilized peptide (continuous line). (C) Differential pulse voltammograms of gold electrodes modified with synthetic PFR + 1 peptide after applying serum from patients with JIA and healthy individuals. (D) Bar plot of the mean peak current showing that the biosensor was able to significantly differentiate patients with JIA and RA from healthy controls.

diagnosis or prognosis, although the rheumatoid factor has been widely used for diagnosing the two polyarticular forms (Duurland and Wedderburn, 2014). The opportunity for the development of novel biomarkers through a subtractive proteomic strategy based on Phage Display (PD) is very attractive and promising for improving JIA diagnosis, since PD libraries contain random peptides that mimic essential properties of either continuous or discontinuous epitopes that do not necessarily present the same amino acid sequences but could mimic the same antibody-binding sites (Geysen et al., 1984). The PD technology has been widely used by our group for the selection of peptides against specific target molecules aiming the discovery of new or increase the action of known epitopes (Araujo et al., 2015b; Feliciano et al., 2014; Santos et al., 2012; Vaz et al., 2015; Alves et al., 2014). Therefore, through a subtractive selection strategy using PD library, we have successfully selected new peptides that could mimic epitopes from antigens of JIA patients and could detect circulating autoantibodies. This strategy led us to 38 different peptides, suggesting that these are immunodominant epitopes involved in the humoral response of JIA patients.

We found that anti-PRF + 1 antibodies were present in 59% of JIA patients, presenting AUC of 0.84, sensitivity of 61% and specificity of 91%. These results represent a very good accuracy for diagnostic tests compared to RF detection. Although rheumatoid factor is one

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Table 2
Sequence and frequency of the selected peptides.

Peptides ID	Amino acids sequence	Frequency (%)
PO1	PGLATNF	1
PO2	SPFWWSD	25
PO3	SPFWWTD	36
PO4	PNPFVLD	1
PO5	LGSVQHT	1
PO6	SPFWWSL	1
PO7	FSPFFWN	1
PO8	WILFFLD	1
PO9	NSPFQLF	1
EO1	SPFFLTP	1
EO2	WNPFLLD	1
EO3	QSPFHLF	1
EO4	SPFWWHQ	3
EO5	NPGWLGS	1
EO6	SAVIKSS	1
EO7	SSHLLSE	1
EO8	LPSRSQT	1
EO9	NNPFQLW	1
EO10	SSFWWTD	1
PRF+1	SSWLPRG	1
PRF+2	WSPFLAP	1
PRF+3	FDPFGWS	2
PRF+4	SPFDWWF	1
PRF+5	NPFFLTA	1
PRF+6	SPNPPLH	1
PRF+7	SSPFFLW	1
PRF+8	SPFHLLP	1
PRF-1	NPFSLLA	1
PRF-2	YFTAPPD	1
PRF-3	DSPFRLW	1
PRF-4	TRPTASH	1
PRF-5	SESPGIA	1
PRF-6	FSPFFAP	1
PRF-7	SPFWLAA	1
PRF-8	SPFFLGP	1
PRF-9	TPFWFLD	1
PRF-10	PAPSRSQ	1
PRF-11	RFGGLIA	1
Total		100

of the widely accepted serological markers of rheumatoid arthritis (Nishimura et al., 2007; Sun et al., 2014), its sensitivity and specificity to JIA is low since it can only be detected in those patients with a late disease onset and those patients presenting joints severely damaged (Petty et al., 2004). The variable potentially associated with anti-PRF+1 antibodies levels was the number of swollen joints in patients with JIA, suggesting that this peptide might be linked to disease activity.

In order to propose a new platform for antibodies detection in JIA, we decided to use differential pulse voltammetry due the fact that this is one of the most sensitive electrochemical techniques, since the current sampling before and after each pulse allows the minimization of charging current (Hennessey et al., 2009). Electrochemical analysis demonstrated that the PRF+1 synthetic peptide was successfully immobilized onto the gold electrode surface. As reported in the literature, gold interacts with sulfur (S) and nitrogen (N) groups (Aryal et al., 2006; Kim et al., 2007) and the PRF+1 peptide contains, among others, two cysteine residues, the conventional terminal amine and a C-terminal amide. These sites can promote the interaction between the peptide and the gold surface and consequently promote the peptide immobilization. The oxidation peak observed in the differential pulse voltammogram in phosphate buffer solution is probably due to the tryptophan residue present in the peptide sequence, since this amino acid presents an oxidation current response in +0.7 V in phosphate buffer (0.1 mol L^{-1}, pH 7.0) in carbon electrodes, and also through differential pulse voltammetry (Enache and Oliveira-Brett, 2013). The result presented in this work shows a similar oxidation peak (+0.69 V), suggesting that it could be due the tryptophan oxidation. Since the electrode was rinsed after each step, this oxidation peak indicates a successful immobilization of the peptide probe onto the gold electrode surface.

The PRF+1 peptide was also able to efficiently discriminate healthy controls from JIA patients through differential pulse voltammetry in potassium ferrocyanide solution, and although serum samples contain many interfering substances, none of them have disturbed the detection process. Potassium ferrocyanide is a redox molecule widely used as indicator in electrochemical analysis to probe surface modifications, since it presents a well-characterized oxidation peak (Hennessey et al., 2009). The interaction between anti-PRF+1 antibodies and the synthetic peptide provides an increase in the concentration of the amino acid residues lysine, histidine and arginine on the gold electrode surface. These amino acid residues are positively-charged leading to the attraction and accumulation of the negatively-charged potassium ferrocyanide on the electrode surface, improving its electronic transference and resulting in increased current peak values by differential pulse voltammetry. The bioelectrode here proposed was able to discriminate samples from JIA patients and healthy children, since the utilized indicator for the molecular recognition between the peptide (probe) and IgG antibodies in the serum sample (target) presented different responses in function. Thus, this bioelectrode based on the anti-PFR+1 antibodies detection presented to be a low cost, fast and reproducible platform and could be used as a biosensor to aid in JIA diagnosis.

In conclusion, we have selected a new target for circulating autoantibodies in patients with JIA that presented high specificity and sensitivity when tested in two different approaches and could be potentially used to aid in diagnosis. Although this peptide is a promising target for antibodies detection, the epitope that this peptide mimics still needs to be identified in a forthcoming study.

Conflict of interest

G.R.A., E.R.V., C.H.M.S., L.R.G., and C.U.V. are co-inventors of a patent protecting the 38 peptide sequences selected in this study. The remaining authors declare that they have no conflicts of interest.

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