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REGULAR ARTICLE

A Rheumatoid arthritis study using Raman spectroscopy

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Abstract Rheumatoid arthritis (RA) is characterized by chronic inflammation of the joints and can lead to a progressive destruction of articular cartilage and bone. In this study, the specificity and sensitivity of the RA diagnostic methods based on the receiver-operating characteristic curves for monitoring C-reactive protein (CRP) and rheumatoid factor (RF) were compared with the Raman spectroscopic diagnostic method developed in this work. Sera from 24 patients with rheumatoid arthritis and from 16 healthy individuals were analyzed to assess the biochemical composition and presence of inflammatory activity by the aforementioned methods. By comparing with the clinical results for specificity and sensitivity from the RF and CRP tests, we show that the overall results from the newly developed Raman method were significantly better, with a specificity of 96%, a sensitivity of 88%, and correctly identifying 92% of the RA and healthy individuals, while the RF test gave a specificity of 100% and a sensitivity of 54%, and the CRP test gave a specificity of 87% and a sensitivity of 58%, respectively.

Keywords Confocal Raman · Rheumatoid arthritis · Serum

Abbreviations

RA	Rheumatoid arthritis
CRP	C-reactive protein
RF	Rheumatoid factor
ACR	American College of Rheumatology
APPs	Acute-phase proteins
NCCLS	National Committee for Clinical Laboratory and Approved Standards
CCD	Charge-coupled device
CaF_2	Calcium fluoride
PCA	Principal components analysis
PC	Principal component
ROC	Receiver-operating characteristic
KS	Kolmogorov and Smirnov
TC	Total cholesterol
r	Correlation coefficient
TG	Total triglycerides
LDL	Low-density lipoprotein
LDA	Linear discriminant analysis
UniFeSP	Universidade Federal de São Paulo
LEVB	Vibrational biomedical spectroscopy laboratory
UniVaP	Universidade do Vale do Paraíba
IP&D	Institute for Research and Development

Dedicated to Professor Akira Imamura on the occasion of his 77th birthday and published as part of the Imamura Festschrift Issue.

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1 Introduction

Recent rheumatoid arthritis (RA) studies have shown that the expression of cytokines and other pro-inflammatory mediators in individuals with RA is primarily responsible for the progressive destruction of articular cartilage and bone [1–4]. RA is a systemic inflammatory disorder characterized by chronic inflammation of unknown causes that primarily affect the joints and extra articulated regions [5–7]. RA strikes approximately 0.5–1.5% of the world's population between the ages of 40 and 60 and is more common in women than in men [6, 7]. RA is a growing public health problem in the USA, requiring a spending of $128 billion dollars per year alone according to recent statistics from the CDC (Centers for Disease Control and Prevention) in Atlanta, Georgia. During the period 2007–2009, there was an increase of approximately 1 million adult cases in USA per year [8].

The difficulties in clinical diagnosis of RA are essentially due to the complexity of this disease. Most cases of RA are unfortunately only identified in the advanced stages. The standard procedure used is based on the patient having at least four of the following seven criteria [established by the American College of Rheumatology (ACR)]: (1) morning stiffness in and around joints lasting at least 1 h before maximal improvement, (2) soft tissue swelling (arthritis) of 3 or more joint areas observed by a physician, (3) swelling (arthritis) of the proximal interphalangeal, metacarpophalangeal, or wrist joints, (4) symmetric swelling (arthritis), (5) rheumatoid nodules, (6) the presence of rheumatoid factor, and (7) radiographic erosions and/or periarticular osteopenia in hand and/or wrist joints [9]. Physicians use patient interview data and the patient anamneses, laboratory examinations, radiographic findings, and clinical tests to document that the patient possesses four or more of the seven criteria and hence has RA. Very recently, the classification scheme for RA has been criticized due to the failure to detect and diagnose RA in its early stages, which has led to a new classification scheme [10].

Within the new scheme, the new criteria are as stated in [10]: classification as "definite RA" is based on the confirmed presence of synovitis in at least one joint, absence of an alternative diagnosis that better explains the synovitis, and achievement of a total score of 6 or greater (of a possible 10) from the individual scores in four domains: number and site of involved joints (score range 0–5), serologic abnormality (score range 0–3), elevated acute-phase response (score range 0–1), and symptom duration (2 levels; range 0–1). This work was based on the old criteria, but the elevated acute-phase response was measured through the presence of C-reactive protein (CRP), which is used in the new one.

However, the early diagnoses are still unavailable because most of these criteria are not specific for RA, and some are present only in the later phases of the disease [11]. Only one of the seven criteria involves the presence of biomarkers, the rheumatoid factors (RFs). RFs are autoantibodies produced in inflamed synovial tissues that form immune complexes in the synovia of patients with RA [12–14].

Nevertheless, the detection of RFs is not absolute, with 20–30% of patients with RA testing negative for its presence [14]. In addition, these antibodies can be produced in other autoimmune diseases, infections, and to a lesser extent, in healthy individuals [12, 15]. The presence of C-reactive protein is also very common in other clinical trials, such as cardiovascular diseases, chronic obstructive pulmonary disease, and cancer [16–19]. The production of large quantities of acute-phase proteins (APPs) during any inflammatory process occurs due to the presence of cytokines, mainly IL-1β, IL-6, and TNF-α. The increased concentration of APPs aids in the diagnosis and is indicative of evaluative inflammatory response (disease progression), but the results have shown low specificity [20–22]. Indeed, there is no consensus in the scientific community about the agent or specific stimulus that triggers the whole chain of events that leads to RA [6, 15].

Raman spectroscopy has been investigated as a promising new tool for diagnosis by measuring the Raman spectra and/or images of biological samples, such as fluids and tissues, for both healthy and diseased states, and using the observed differences in the individual spectra and images as characteristic biomarkers. The published results have shown high potential in distinguishing normal and abnormal characteristics of the samples [23–25]. In this study, the sera of healthy and patients with RA were analyzed by Raman spectroscopy in the 775–1,775 cm^{-1} region. The functional group frequencies and Raman intensities assigned to the amide groups in proteins and the secondary structural elements and hydration and aggregation states of the proteins, immunoglobulins, and lipids were correlated with clinical signals and symptoms as well as the biochemical changes present in the rheumatic processes. The diagnoses were also performed with traditional clinical tests (CRP and RF), which were used to confirm the diagnoses made with the new Raman spectroscopic method reported in this work.

2 Experimental details

2.1 Blood samples (collection and storage)

Sera from 40 women from the ambulatory at UniFeSP were collected in accordance with the requirements and

recommendations of the National Committee for Clinical Laboratory and Approved Standards (NCCLS) [26] and subsequently taken to the Vibrational Biomedical Spectroscopy Laboratory (LEVB) of the Institute for Research and Development (IP&D) of the Universidade do Vale do Paraíba (UniVaP) where the Raman and clinical analyses were undertaken. A total of 16 samples were from healthy donors and 24 from patients with RA defined according to criteria of the ACR from 1987 [9]. The following inclusion and exclusion parameters were preset for both groups. In the healthy group, 30- to 60-year-old women were recruited, without related chronic diseases. The RA group women included only patients with confirmed diagnoses. The procedure for collection and storage used involved the following steps: (1) venous blood samples were collected through an antecubital venipuncture of the right arm; (2) the blood was drawn into sterile and gel-barrier collection tubes (BD Vacutainer SST Advance Tube, 5 mL, Gold); (3) the blood was centrifuged at 4,000*g* for 6 min; (4) and finally, the sera were stored in Nalgene® tubes at −20 °C.

2.2 Raman spectroscopy

The samples were warmed to room temperature, homogenized by a mixer (Vortex AP 56–Phoenix) at 2,000*g* for 10 s, and then a drop (10 μL) was deposited on a calcium fluoride (CaF_2) window and lyophilized for 45 min (Eppendorf Concentrator 5301) to concentrate the sample.

The Raman spectrum acquisition was performed using a confocal system (Horiba Jobin–Yvon) connected to an EspectraPro spectrometer® (PI-Acton 2500i) equipped with a charge-coupled device (CCD) detector (Spec10, Princeton). The excitation source was a 785-nm diode laser delivering 10 mW of power to the serum sample. Two spectra were collected for each sample for 25 s spectra collection times with three accumulations. All spectra were input into the WinSpec® program and therewith underwent the following signal processing: (1) a baseline correction was done using a Matlab® routine which fit the background Raman scattering to a polynomial function [27]; (2) the average spectrum was calculated for each sample; (3) the spectra were smoothed using adjacent averaging to remove the background noise, (4) the spectra were normalized by dividing by the value at 1,430 cm^{-1}, which corresponding to the maximum of spectra intensity; (5) and finally, all spectra were mean centered for statistic analysis using the Minitab® program.

After this first step (normalization followed by mean centering), the data were analyzed using the principal components analysis (PCA) module within the Minitab® program. In this study, the first six principal components (PC1-PC6) were used to perform the linear discriminant analysis to classify the RA and *N* groups according to the pathological reports. The areas of the main spectra peaks were calculated and analyzed through a parametric test.

The distribution of the areas calculated was assumed to be Gaussian, and this assumption/hypothesis was verified using the Kolmogorov–Smirnov test for normal distributions. The one-way ANOVA test was used to compare the group means with each other, which had a significance level below 0.05 (Minitab® program). This test is used to compare groups of samples of different sizes using the *F* statistic [28], where *F* is the ratio of the two different estimates with the common population variance. For the calculations with different sample sizes, one applies the factor F given by standard Eq. 1.

$$F = \frac{\frac{\sum n_i(\bar{X}_i - \overline{\overline{X}})^2}{k-1}}{\frac{\sum (n_i-1)S_i^2}{\sum (n_i-1)}} \quad (1)$$

where $\bar{X}$ is the average values in the *i*th sample; $\overline{\overline{X}}$ is the average values of all samples combined; *k* is the number of population means being compared; n_i is the number of values in the *i*th sample; and S_i^2 is the variance of the values in the *i*th sample.

2.3 Clinical analysis

The RF and CRP analyses were done by indirect agglutination using latex coated with human IgG and with a high-purity, anti-CRP monoclonal antibody (2900L-909024AB of Wama Diagnostic® Kit), respectively. These tests were chosen because they are the ones most frequently used in RA trials [29, 30].

2.4 Medication and alimentary diet

In this study, there was neither food restriction nor a standardized diet for all individuals involved. Blood was collected in the morning, at which time all the volunteers reported having had a light meal.

Individuals in both groups were using specific medications. In the RA group, the types of drugs and their associations depended on the RA stage, activity, and disease severity. Foremost among them those being taken were disease-modifying anti-rheumatic drugs (DMARDs), corticosteroids (used at physiological concentrations: quantities normally found in the body), nonsteroidal anti-inflammatory drugs (NSAIDs), and antihypertensive drugs. In the control group, 40% of the subjects reported using antihypertensive drugs, mainly used to control the blood pressure [31, 32].

3 Results and discussions

The results are presented in two parts. First, all samples were analyzed using the standard clinical tests, and the values of specificity and sensitivity were determined. Second, all samples were analyzed using the Raman methodology developed in this work, and the values of specificity and sensitivity were determined. In addition, we provide a biological interpretation for the Raman data we have collected.

3.1 Clinical analysis

Inflammatory activity was measured for all samples by using the standard RF and CRP tests. All sample positives were tested in duplicate, the so-called A and B samples. Laboratory tests on the B samples were made to confirm the results of laboratory tests for all A samples that showed a positive response with an agglutination test which was visible to the naked eye. Figure 1 shows a comparison of the specificity and sensitivity for these tests, which were determined using receiver-operating characteristic curves (ROCs) [33].

The RF and CRP specificity values were 1.00 and 0.87, respectively, while the RF and CRP sensitivity values were 0.58 and 0.54, which are significantly lower. This result can be explained by the limitations of the RF and CRP tests. Clear serum agglutination for samples with concentrations below 6 mg/L of CRP protein of acute phase and below 8 UL/mL of RF was not seen. These results are in agreement with other works where it was shown that the RF test has a higher specificity but a lower sensitivity [34–36].

CRP is a marker used to show an acute inflammatory response during an infection, tissue damage (injury), or neoplasia [37]. However, the presence of these acute-phase proteins is also present in healthy individuals, because of any micro-inflammation in the body [38].

3.2 Raman spectroscopy

Figure 2 shows the average Raman spectrum for the *N* and RA groups. In order to help identify the slight differences, a residual line was created by subtracting the RA from the N spectra. The negative and positive fluctuations were represented by the dark filled area, indicating the biochemical variations. Figure 2 shows small differences in the molecular structure of the sera of both groups. Based on this evidence, inferential statistics was used to highlight the biochemical variations of the serum.

The principal components analysis was performed in the range of 775–1,775 cm^{-1} by a covariance matrix. Figure 3 displays the loading plot for the first six PCs, which correspond to the variation of PCs as a function of Raman shift. The six first PCs were considered based on the eigenvalues, and data proportion that were, respectively, PC1 11.87 and 29.7%; PC2 8.99 and 22.5%; PC3 4.93 and 12.3%; PC4 3.21 and 8%; PC5 2.6 and 6.5%; PC6 1.3 and 3.4%. PC1, PC2, and PC3 represent 64.5% of the total data variance.

The spectra were analyzed by linear discriminant analysis (LDA) using the first six principal components and a cross-validation method, which works by omitting each observation one at a time, recalculating the classification function using the remaining data, and then classifying the omitted observation. The classification role was based on medical reports with thorough anamneses, laboratory, and radiographic exams according to ACR rules [9, 15]. The results are shown in Table 1.

The result shows a total correct proportion of 92.5%. There was one false positive for the *N* group and two false negatives for the RA group. Figure 4 shows the ROC curve

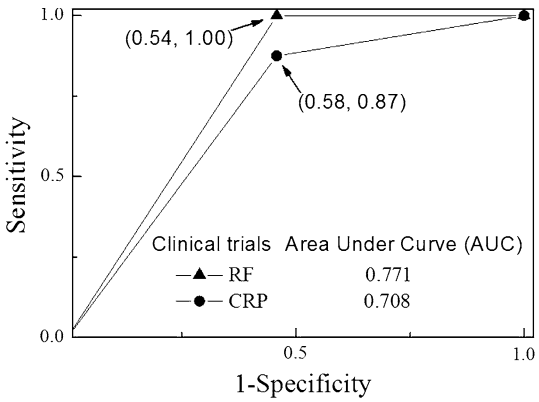

Fig. 1 Specificity and sensitivity as function of the clinical trials RF (*filled triangle*) and CRP (*filled circle*)

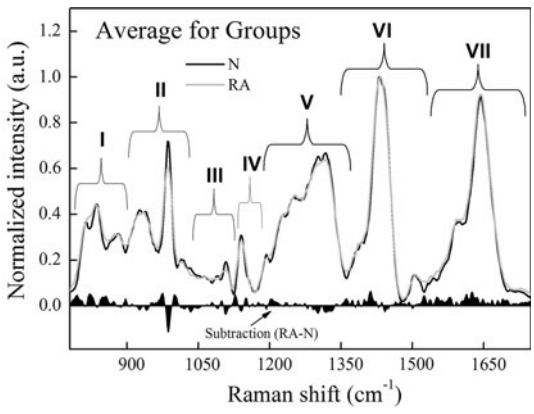

Fig. 2 Spectra averages for normal and rheumatic groups and the spectra differences given by the *subtraction line*

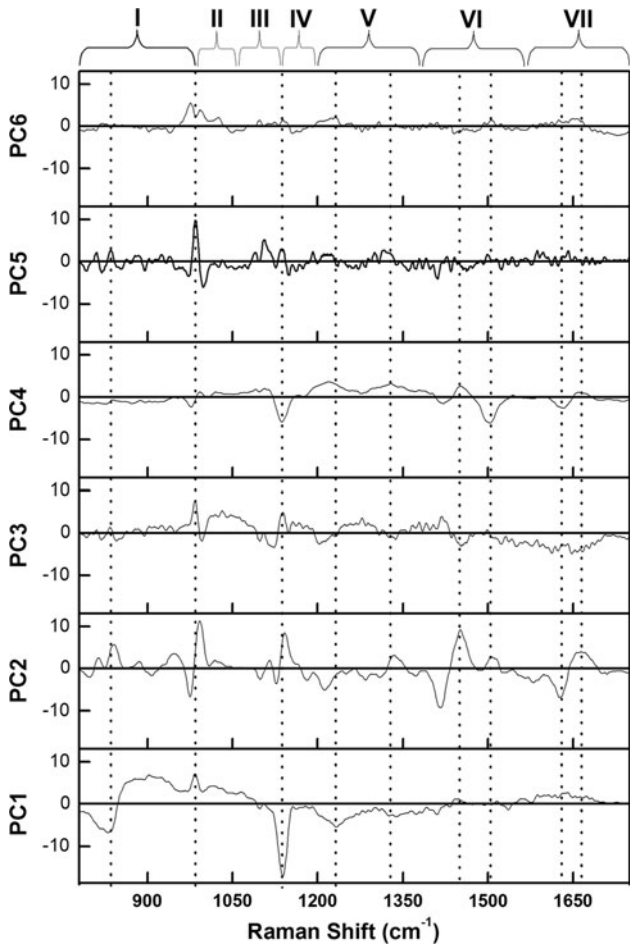

Fig. 3 Loading plot for the six first principal components of the total data

Table 1 Summary classification of LDA

Classification with cross-validation		
Put into group	True group	
	N	RA
N	14	1
RA	2	23
Total number	16	24
Number correct	14	23
Proportion (%)	87.5	95.8

$N = 40$, N correct $= 37$, Proportion correct $= 92.5\%$

that was calculated using the values of the centroid distances given by LDA.

The Raman results gave the best values overall for specificity and sensitivity which values of 96 and 88%, respectively. Based on these results, the optical Raman spectroscopy can be utilized for the differential diagnosis of RA. The information obtained by this technique is extremely important for the development of a spectral library for the classification of rheumatic diseases.

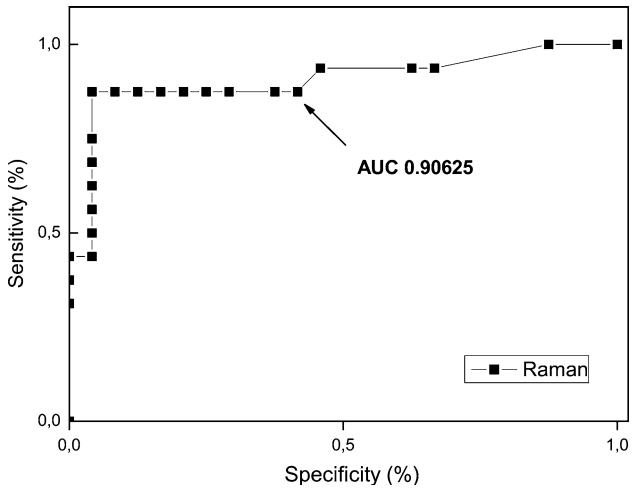

Fig. 4 ROC curve from Raman data based on LDA

3.2.1 *Medications and diet*

Both dietary and drug regimes have been reported to have possible interfering affects on the Raman spectra if not properly accounted for in the analysis. Below, we discuss and refer to some studies documenting some of these effects which also suggest that changes in concentration and conformation of proteins and lipids in human serum may be a predictor of RA.

Disease-modifying anti-rheumatic drugs (DMARDs), also known as antimetabolites such as methotrexate (MTX) and leflunomide, were the main drugs used by patients with RA in this study. MTX is a drug with anti-folate properties commonly used in the treatment of RA and may be associated with other drugs such as leflunomide. The long-term use of MTX causes gastrointestinal intolerance, mucositis, alopecia, and more severe/rare complications such as hematological abnormalities, hepatotoxicity, and pulmonary toxicity [39–42]. Leflunomide inhibits pyrimidine synthesis and regulates the proliferation of CD4 [43], may cause nausea, diarrhea, alopecia, hypertension, and may cause elevated levels of some enzymes in the liver [39]. However, both drugs are not associated with dyslipidemia [39]. Park et al. [44] describe in his study that liver dysfunction is not common even at high doses of MTX, since these risk factors have been controlled in the development of this drug. Park et al. [45] suggest that lipid changes take place before the use of these medications and hence are not due to DMARDs. In this study, laboratory monitoring of complete blood count, liver function tests (AST and ALT), and monitoring of creatinine were used to check for possible side effects in the patients with RA. Kermani and

Luthra [46] say that the use of folic acid during treatment with MTX reduces its side effects. The combination of these drugs is being taken by many of our rheumatic patients.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac and ketoprofen have potent anti-inflammatory effect, analgesic with antipyretic properties, are well absorbed orally, and dissolve in the intestinal fluid [47]. Dipyrone is a potent analgesic drug that causes minor adverse effects [48].

Corticosteroids such as prednisone are used only in cases of pain and at physiological doses (quantities normally found in the body) in combination with supplemental calcium carbonate ($CaCO_3$) and vitamin D (cholecalciferol). The use of corticosteroids is associated with serious dysfunctions, such as hypertension, fluid retention, gastrointestinal disturbances, increased blood glucose, osteoporosis, and dyslipidemia [39]. However, in the RA group, these medications were prescribed only at physiological dosages during painful crises.

Another group of drugs considered in our study are the antihypertensives, such as β-blockers (atenolol), ACE inhibitors (captopril and enalapril), diuretics (hydrochlorothiazide), and angiotensin II receptor blockers. In a 1999 study, Papadakis stated that antihypertensive drugs do not cause significant changes in lipid levels [49]. In 1988, Krone suggested that α, β-adrenergic antagonists and calcium antagonists affect lipid metabolism, but these changes have not been clearly elucidated which suggests that previous analyses should be reinvestigated to exclude any preexisting hyperlipidemias [50]. In 2007, Hadda reported that lipid levels in patients with RA tend to normalize with the control of the disease activity with medications [51]. In 2009, Steiner showed that DMDC has beneficial effects on both inflammation and maintaining the proper lipid levels and hence limiting the risk of cardiovascular disease [52]. In 2008, Seven showed that the increase in lipids, proteins, markers of DNA oxidation and antioxidant factors may indicate the activity of RA, showing that lipid peroxidation can serve as marker of RA [53]. These studies corroborate the findings highlighted in our study that lipid abnormalities found by Raman spectroscopy can predict the activity of RA.

3.2.2 *Biochemical interpretation of the spectra*

Table 2 shows the main Raman assignments of molecules in human blood, which has a column to distinguish the different blood constituents analyzed by various authors [55–61].

The Raman spectra were divided in several parts based on the molecules assignments (Table 2) and also on the variance of PCs (Fig. 3). The Figs. 2 and 3 have the regions markers, separating the following spectral ranges: (I) 776–900 cm^{-1}; (II) 902–1,049 cm^{-1}; (III) 1,051–1,098 cm^{-1}; (IV) 1,100–1,180 cm^{-1}; (V) 1,181–1,370 cm^{-1}; (VI) 1,372–1,599 cm^{-1}; (VII) 1,601–1,776 cm^{-1}.

In order determine the statistic significance of each region, the areas were calculated and analyzed by the statistical parametric test [54]. The parametric test assumes that the both the groups RA and *N* have a normal distribution. For each spectral region, the normality distribution of the data was checked by descriptive statistics using the method of Kolmogorov and Smirnov the so-called KS test

Table 2 The main Raman assignments of molecules in the human blood

Range (cm^{-1})	Human blood	Molecules assignments	References
510–1,200	Sera	Lipids (HDL and LDL)	[55]
510–1,320	Blood and sera	Glucose	[55, 56]
510–1,800	Blood and sera	Triglycerides	[55, 56]
550–1,800	Sera	Creatinine	[55]
565–1,746	Sera	Cholesterol	[56]
720–1,602	Blood and sera	Albumin	[55, 56]
744/760/1,342/1,448	Blood and sera	Tryptophan	[57, 58]
829–851	Sera	Tyrosine	[57, 59]
1,000/1,003	Blood and sera	Phenylalanine	[57, 58]
1,000–1,800	Sera	Globulin	[55]
940–1,320	Plasma	∝ hélix	[57]
960	Plasma	Random coil	[57]
990/1,240	Plasma	β sheet	[57]
1,230–1,309	Plasma and sera	Amide III	[60, 61]
1,542/1,620	Dried blood	Hemoglobin	[58]
1,645–1,680	Plasma	Amide I	[60]
1,654	Sera	Amide I	[61]

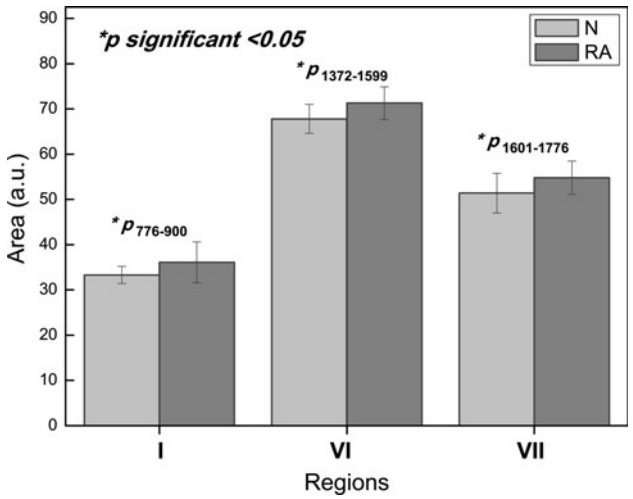

Fig. 5 Statistical results of the significant spectral ranges of the full spectra

[28]. All the regions were analyzed using the ANOVA method [28]. Regions II, III, IV, and V have many variations as shown in Fig. 2 (RA-*N* subtraction line), but these are not statistically relevant due to the high data variance. The variations in these regions are due to other variables (see Sects. 2.6, 3.2.1) other than those specific for RA. Regions I, VI, and VII have variations which are statistically significant and are due specifically to RA. The calculated areas for these regions with the *p* significant values are shown in the Fig. 5.

3.2.2.1 Region I (776–900 cm^{-1}) This region has many assignments from the molecules such as lipids, glucose, triglycerides, tryptophan, creatinine, cholesterol, albumin, and tyrosine [55–59]. The most abundant plasma proteins are the serum albumins that contribute to the transport and regulatory processes of the cells. Tyrosine phosphorylation is important for the regulation of several physiological processes such as cell proliferation, migration, and differentiation [62]. However, there are studies that claim that different tyrosines participate in the pathophysiology of RA and that the inhibitors of these tyrosines contribute to the reduction of symptoms and disease activity [63]. There is evidence that tyrosine kinases inhibitors have the ability to inhibit cytokine production [63]. Tryptophan is required for the biosynthesis of proteins and is a precursor for several biologically important compounds. In vivo, enhanced cytokine-induced degradation of tryptophan is observed whenever a cellular immune response is induced [64]. With respect to the tryptophan studies, one has concluded that patients with RA have a deficiency in the concentration of free peptides, which would result in less protection of the connective tissue during inflammatory attacks [65]. The variations of the calculated area could indicate a greater involvement of the tyrosine doublet at 829–851 cm^{-1} in patients with RA that perpetuates the inflammatory process.

Cytokines are local protein mediators, involved in almost all important biological activities (cell growth, activation, inflammation, immunity, and differentiation) [2]. Thus, there is agreement between several works that cytokines play an important role in autoimmune diseases, like RA [2, 3, 21]. Studies suggest that the increase in cytokines such as IL-1α, IL-8, and TNF-α is not derived from circulating monocytes of patients with RA. They believe that the source of these cytokines is the inflamed joints [2, 66]. In the case of RA, the increase in APP's, IL-I, TNF-α, and IL-6 concentrations in the circulatory system is usually associated with a severe stage of disease progression [67]. Nevertheless, the cytokines vibrational modes are still unknown. Many authors have been studying the sera by Fourier Transform Infrared Spectroscopy (FT-IR), and modes in this region have been correlated with the CO stretching modes (900–1,300 cm^{-1}) of glucose and lactate [68–70].

3.2.2.2 Region VI (1,372–1,599 cm^{-1}) This region has the contributions of the albumin mode (720–1,602 cm^{-1}) [55, 56] as shown in Table 2. Albumin is involved on the transport process of metal ions, fatty acids, bilirubin, and drugs in the blood. Albumin is also involved in the pharmacokinetics of various therapeutic drugs that can be bound to the protein. Changes in vibrational modes of region VI may be due to medications used by patients with RA [71, 72]. Pezolet et al. [73] had described the region of the 1,406 cm^{-1} for COO^- stretching vibration modes of immunoglobulin G (IgG) that are composed of peptide chains and tryptophan (1,554–1,584 cm^{-1}). Deléris et al. [69, 70] have reported this region in the FT-IR study, and molecular assignments were to COO^- stretching, CH_2 and CH_3 bending of amino acids, fatty acids, phospholipids, and triglycerides.

3.2.2.3 Region VII (1,601–1,776 cm^{-1}) This region has the contribution of amide I vibrational modes. The amide I usually reflects hydrogen bonding in the various secondary structural elements in proteins, for example the hydrogen-bonding pattern of the C=O groups both within the albumin protein (intramolecular hydrogen bonding) and with other proteins and the environment (intermolecular hydrogen bonding) [74]. Fraile et al. [75, 76] reported changes in the region of 1,630–1,695 cm^{-1}, corresponding to amide I region, on the addition of lipids to albumin which reflect not only changes in the hydrogen bonding but also of the presence of changes in the secondary structural elements. Dotzlaw et al. [77] described in his study that the expression of proteins can serve as a baseline to monitor the effectiveness of therapy and be of diagnostic value for the

classification of autoimmune diseases according to molecular pathophysiology.

Extensive conformational and compositional analyses of serum and other biological solutions by optical spectroscopy have been reported, for example, proteins (albumin-HSA), peptides, amides, carbohydrates, and glucose [69, 70, 78–81]. Raman spectroscopy is an important analytical method for the identification of specific molecules [82] and can be used for diagnostic or prognostic value for verification of chemical changes [82, 83]. One of the limitations found is the lack of standardization of the spectral bands of specific molecules present in serum. New markers need to be scored to try to establish a relationship between the serological analysis and optical spectroscopy through chemicals entities, which do not suffer the interference of dietary factors or drug interactions.

4 Conclusions

Our work here lends further support to other similar studies in demonstrating that Raman spectroscopy may be of interest as a diagnostic tool for RA by determining not only the presence of biomolecules such as proteins, amines, lipids and immunoglobulins but also changes in the molecular and structural composition and chemicals entities. The results from the statistical analysis showed that this technique is extremely promising for use in the diagnosis of rheumatic disease. However, one still needs to understand more about the constituents in isolation from serum in order to establish more precise regions for compounds present in human serum. Future studies are needed to clarify the effectiveness of the technique for early diagnosis of RA using the recently developed new criteria.

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References

1. Jazayeri JA, Carrol GJ, Vernallis AB (2010) Interleukin-6 subfamily cytokines and rheumatoid arthritis: role of antagonists. Int Immunopharmacol 10:1–8
2. Feldmann M, Brennan FM, Maini RN (1996) Role of cytokines in rheumatoid arthritis. Ann Rev Immunol 14:397–440
3. Carteron NL (2000) Cytokines in rheumatoid arthritis: trials and tribulations. Mol Med Today 6:315–323
4. Walsmith J, Roubenoff R (2002) Cachexia in rheumatoid arthritis. Int J Cardiol 85:89–99
5. Van Boekel M, Vossenaar ER, Van Den Hoogen FHJ, Van Venrooij WJ (2002) Autoantibody systems in rheumatoid arthritis: Specificity, sensitivity and diagnostic value. Arthritis Res 4:87–93
6. Khurana R, Berney SM (2005) Clinical aspects of rheumatoid arthritis. Pathophysiology 12:153–165
7. Chang J, Kavanaugh A (2005) Novel therapies for rheumatoid arthritis. Pathophysiol 12:217–225
8. Cheng YJ, Hootman JM, Murphy LB, Langmaid GA, Helmick CG (2010) Prevalence of doctor-diagnosed arthritis and arthritis-attributable activity limitation—United States, 2007–2009. Morbidity and mortality weekly report 59:1261–1265. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5939a1.htm?s_cid=mm5939a1_w
9. Arnett FC, Edworthy SM, Bloch DA, Mcshane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA, Mitchell DM, Neustadt DH, Pinals RS, Schaller JG, Sharp JT, Wilder RL, Hunder GG (1988) The American rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31:315–324
10. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JMW, Hobbs K, Huizinga TWJ, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Ménard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, Tak PP, Upchurch KS, Vencovsky J, Wolfe F, Hawker G (2010) 2010 Rheumatoid arthritis classification criteria. Arthritis Rheum 62:2569–2581
11. Vossenaar ER, Van Venrooij WJ (2004) Anti-CCP antibodies, a highly specific marker for (early) rheumatoid arthritis. Clin Applied Immunol Rev 4:239–262
12. Sutton B, Corper A, Bonagura V, Taussig M (2000) The structure and origin of rheumatoid factors. Immunol Today 4:177–183
13. Chaiamnuay S, Louis Bridges SJ (2005) The role of B cells and autoantibodies in rheumatoid arthritis. Pathophysiology 12:203–216
14. Mewar D, Wilson AG (2006) Autoantibodies in rheumatoid arthritis: a review. Biomed Pharmacother 60:648–655
15. Nijenhuis S, Zendman AJW, Vossenaar ER, Pruijin Ger JM, vanVenrooij W (2004) Autoantibodies to citrullinated proteins in rheumatoid arthritis: clinical performance and biochemical aspects of an RA-specific marker. Clin Chim Acta 350:17–34
16. Ottaviani S, Mariani F, Ferrarotti I, Luisetti M (2011) C reactive protein and alpha 1-antitrypsin: relationship between levels and gene variants. Transl Res doi:10.1016/j.trsl.2010.12.014. http://dx.doi.org/10.1016/j.trsl.2010.12.014
17. Sjowall C, Wettero J (2007) Pathogenic implications for autoantibodies against C-reactive protein and other acute phase proteins. Clin Chim Acta 378:13–23
18. Levinson SS (2005) Brief review and critical examination of the use of hs-CRP for cardiac risk assessment with the conclusion that it is premature to use this test. Clin Chim Acta 356:1–8
19. Heikkila K, Silander K, Salomaa V, Jousilahti P, Koskinen S, Pukkala E, Perola M (2011) C-reactive protein-associated genetic variants and cancer risk: findings from FINRISK 1992, FINRISK 1997 and Health 2000 studies. Eur J Cancer 47:404–412
20. Marnell L, Mold C, Du Clos TW (2005) C-reactive protein: ligands, receptors and role in inflammation. Clin Immunol 117:104–111
21. Cassim B, Mody G, Bhoola KD (2002) Kallikrein cascade and cytokines in inflamed joints. Pharmacol Ther 94:1–34
22. van Riel PLCM, Fransen J (2007) Established rheumatoid arthritis: clinical assessments. Best Pract Res Clin Rheumatol 21:807–825
23. Moreno M, Raniero L, Arisawa EAL, Santo AME, Santos EAP, Bitar RA, Martin AA (2010) Raman spectroscopy study of breast disease. Theor Chem Acc 125:329–334
24. Esmonde-White KA, Mandair GS, Raaii F, Jacobson JA, Miller BS, Urquhart AG, Roessler BJ, Morris MD (2009) Raman

spectroscopy of synovial fluid as a tool for diagnosing osteoarthritis. J Biomed Opt 14:034013
25. Hanlon EB, Manoharan R, Koo TW, Shafer KE, Motz JT, Fitzmaurice M, Kramer JR, Itzkan I, Dasari RR, Feld MS (2000) Prospects for in vivo Raman spectroscopy. Phys Med Biol 45:R1–R59
26. CLSI (2003) Clinical and laboratory standards institute quality manual, 3rd edn. CLSI, Wayne http://www.clsi.org/Content/NavigationMenu/Resources/QualityManualThirdEdition.pdf
27. Lieber CA, Mahadevan-Jansen A (2003) Automated method for subtraction of fluorescence from biological Raman spectra. Appl Spectrosc 57:320–340
28. Triola MF (2009) Elementary statistics plus MyStatLab student access kit, 11th edn. Addison-Wesley, Rio de Janeiro, ISBN-13:978-0321570895
29. Van Venrooij WJ, Van Beers JJBC, Pruijn GJM (2008) Anti-CCP antibody, a marker for the early detection of rheumatoid arthritis. The year in immunology. Ann N Y Acad Sci 1143:268–285
30. Heidari B, Heidari P, Tayebi ME (2007) The value of changes in CRP and ESR for predicting treatment response in rheumatoid arthritis. APLAR J Rheumatol 10:23–28
31. Lin F, Chen L, Liang R, Zhang Z, Wang J, Cai M, Li Y (2011) Pilot-scale production of low molecular weight peptides from corn wet milling byproducts and the antihypertensive effects in vivo and in vitro. Food Chem 124:801–807
32. Perret-Guillaume C, Genet C, Herrmann FR, Benetos A, Hurst SA, Vischer UM (2011) Attitudes and approaches to decision making about antihypertensive treatment in elderly patients. Am Med Dir Assoc 12:121–128
33. Fawcett T (2006) An introduction to ROC analysis. Pattern Recognit Lett 27:861–874
34. Bizarro N, Mazzanti G, Tonutti E, Villalta D, Tozzoli R (2001) Diagnostic accuracy of the anti-citrulline antibody assay for rheumatoid arthritis. Clin Chem 47:1089–1093
35. Hoffman IEA, Peene I, Pottel H, Union A, Hulstaert F, Meheus L, Lebeer K, De Clercq L, Schatteman L, Poriau S, Mielants H, Veys EM, De Keyser F (2005) Diagnostic performance and predictive value of rheumatoid factor, anti-citrullinated peptide antibodies, and the HLA shared epitope for diagnosis of rheumatoid arthritis. Clin Chem 51:261–263
36. Vander Cruyssen B, Peene I, Cantaert T, Hoffman IEA, De Rycke L, Veys EM, De Keyser F (2005) Anti-citrullinated protein/peptide antibodies (ACPA) in rheumatoid arthritis: specificity and relation with rheumatoid factor. Autoimmun Rev 4:468–474
37. Pepys MB, Hirschfield GM (2003) C-reactive protein: a critical update. J Clin Investig 111:1805–1812
38. Rogowski O, Vered Y, Shapira I, Hirsh M, Zakut V, Berliner S (2005) Introducing the wide range C-reactive protein (wr-CRP) into clinical use for the detection of microinflamation. Clin Chim Acta 358:151–158
39. Meyer KC, Decker C, Baughman R (2010) Toxicity and monitoring of immunosuppressive therapy used in systemic autoimmune diseases. Clin Chest Med 31:565–588
40. Stamp L, Roberts R, Kennedy M, Barclay M, O'Donnell J, Chapman P (2006) The use of low dose methotrexate in rheumatoid arthritis-are we entering a new era of therapeutic drug monitoring and pharmacogenomics. Biomed Pharmacother 60:678–687
41. Alarcón GS (2000) Methotrexate use in rheumatoid arthritis. A clinician's perspective. Immunopharmacol 47:259–271
42. Borchers AT, Keen CL, Cheema GS, Gershwin ME (2004) The use of methotrexate in rheumatoid arthritis. Semin Arthritis Rheumatol 34:465–483
43. Kremer JM (1999) Methotrexate and leflunomide: biochemical basis for combination therapy in the treatment of rheumatoid arthritis. Semin Arthritis Rheumatol 29:14–26
44. Park S-H, Choe J-Y, Kim S-K (2010) Assessment of liver fibrosis by transient elastography in rheumatoid arthritis patients treated with methotrexate. Joint Bone Spine 77:588–592
45. Park Y-B, Choi HK, Kim M-Y, Lee W-K, Song J, Kim D-K, Lee S-K (2002) Effects of antirheumatic therapy on serum lipid levels in patients with rheumatoid arthritis: a prospective study. Am J Med 113:188–193
46. Kermani TA, Luthra HS (2008) Folate supplementation and methotrexate in treatment of rheumatoid arthritis. Indian J Rheumatol 3:77–79
47. Espinosa-Mansilla A, La Peña AM, Cañada-Cañada F, Gómez DG (2005) Determinations of xuoroquinolones and nonsteroidal anti-inflammatory drugs in urine by extractive spectrophotometry and photoinduced spectrofluorimetry using multivariate calibration. Anal Biochem 347:275–286
48. Levy M, Zylber-Katz E, Rosenkranz B (1995) Clinical pharmacokinetics of dipyrone and its metabolites. Clin Pharmacokinet 28:216–234
49. Papadakis JA, Ganotakis ES, Jagroop IA, Mikhailidis DP, Winder AF (1999) Effect of hypertension and its treatment on lipid, lipoprotein(a), fibrinogen, and bilirubin levels in patients referred for dyslipidemia. Am J Hypertens 12:673–681
50. Krone W, Nagele H, Hamburg MD (1988) Effects of antihypertensives on plasma lipids and lipoprotein metabolism. Am Heart J 116:1729–1734
51. Hadda V, Handa R, Aggarwal P, Lakshmy R, Kumar U, Pandey RM (2007) Disease activity and lipids in rheumatoid arthritis: a prospective study. Indian J Rheumatol 4:137–140
52. Steiner G, Urowitz MB (2009) Lipid profiles in patients with rheumatoid arthritis: mechanisms and the impact of treatment. Semin Arthritis Rheumatol 38:372–381
53. Seven A, Guzel S, Aslan M, Hamuryudan V (2008) Lipid, protein, DNA oxidation status in rheumatoid arthritis. Clin Biochem 14:538–543
54. Pajevic S, Basser PJ (2003) Parametric and non-parametric statistical analysis of DT-MRI data. J Magn Reson 161:1–14
55. Qi D, Berger AJ (2007) Chemical concentration measurement in blood serum and urine samples using liquid-core optical fiber Raman spectroscopy. Appl Opt 46:1726–1734
56. Berger AJ, Koo T-W, Itzkan I, Horowitz G, Feld MS (1999) Multicomponent blood analysis by near-infrared Raman spectroscopy. Appl Opt 38:2916–2926
57. Dàvila E, Parés D, Howell N (2007) Studies on plasma protein interactions in heat-induced gels by differential scanning calorimetry and FT-Raman spectroscopy. Food Hydrocoll 21:1144–1152
58. Virkler K, Lednev IK (2010) Raman spectroscopic signature of blood and its potential application to forensic body fluid identification. Anal Bioanal Chem 396:525–534
59. Rohleder D, Kocherscheidt G, Gerber K, Kiefer W, Kohler W, Mocks J, Petrich W (2005) Comparison of mid-infrared and Raman spectroscopy in the quantitative analysis of serum. J Biomed Opt 10:031108. doi:10.1117/1.1911847
60. Herrero AM, Cambero MI, Ordóñez JA, La Hoz L, Carmona P (2009) Plasma powder as cold-set binding agent for meat system: rheological and Raman spectroscopy study. Food Chem 113:493–499
61. Fleury F, Ianoul A, Berjot M, Feofanov A, Alix AJP, Nabiev I (1997) Camptothecin-binding site in human serum albumin and protein transformations induced by drug binding. FEBS Lett 411:215–220
62. Xie Y, Zhang D, Jarori GK, Davisson VJ, Ben-Amotz D (2004) The Raman detection of peptide tyrosine phosphorylation. Anal Biochem 332:116–121
63. Tristano AG (2009) Tyrosine kinases as targets in rheumatoid arthritis. Int Immunopharmacol 9:1–9

64. Schrocksnadel K, Wirleitner B, Winkler C, Fuchs D (2006) Monitoring tryptophan metabolism in chronic immune activation. Clin Chim Acta 364:82–90
65. McArthur JN, Dawkins PD, Smith MJH, Hamilton EBD (1971) Mode of action of antirheumatic drugs. Br Med J 2:667–679
66. Rodenburg RJT, Van de Hoogen FHJ, Van de Putte LBA, Van Venrooij WJ (1998) Peripheral blood monocytes of rheumatoid arthritis patients do not express elevated TNF-α amd IL-8 mRNA levels. A comparison of monocyte isolation procedures. J Immunol Meth 221:169–175
67. Badolato R, Oppenheim JJ (1996) Role of cytokines, acute-phase proteins, and chemokines in the progression of rheumatoid arthritis. Semin Arthritis Rheum 26:526–538
68. Petibois C, Melin AM, Perromat A, Cazorla G, Déléris G (2000) Glucose and lactate concentration determination on single microsamples by Fourier-transform infrared spectroscopy. J Lab Clin Med 135:210–215
69. Petibois C, Cazorla G, Cassaigne A, Déléris G (2001) Plasma protein contents determined by Fourier-transform infrared spectrometry. Clin Chem 47:730–738
70. Déléris G, Petibois C (2003) Applications of FT-IR spectrometry to plasma contents analysis and monitoring. Vib Spectrosc 32:129–136
71. Barnaby OS, Cerny RL, Clarke W, Hage DS (2011) Comparison of modification sites formed on human serum albumin at various stages of glycation. Clin Chim Acta 412:277–285
72. Rondeau P, Bourdon E (2011) The glycation of albumin: structural and functional impacts. Biochim doi:10.1016/j.biochi.2010.12.003. http://dx.doi.org/10.1016/j.biochi.2010.12.003
73. Pezolet M, Pigeon-Gosselin M, Coulombe L (1976) Laser Raman investigation of the conformation of human immunoglobulin G. Biochim Biophys Acta 453:502–512
74. Sane SU, Cramer SM, Przybycien TM (1999) A holistic approach to protein secondary structure characterization using amide I band Raman spectroscopy. Anal Bioch 269:255–272
75. Fraile MV, Blanco-Melgar M, Munõz M, Rodriguez GL, Gallego-Nicasio JA, Carmona P (2003) Structure and interactions of albumin-lipid systems as studied by infrared spectroscopy. J Mol Struct 651–653:231–236
76. Sadler PJ, Tucker A, Viles JH (1994) Involvement of a lysibe residue in the N-terminal Ni2+ and Cu2+ binding site of serum albumins. Comparison with Co^{2+}, Cd^{2+} and $A1^{3+}$. Eur J Biochem 220:193–200
77. Dotzlaw H, Schulz M, Eggert M, Neeck G (2004) A pattern of protein expression in peripheral blood mononuclear cells distinguishes rheumatoid arthritis patients from healthy individuals arthritis patients from healthy individuals. Biochim Biophys Acta 1696:121–129
78. Jalkanen KJ, Suhai S (1996) N-acetyl-L-alanine N′-methylamide: a density functional analysis of the vibrational absorption and vibrational circular dichroism spectra. Chem Phys 208:81–116
79. Diem M, Griffiths PR, Chalmers JM (eds) (2008) Vibrational spectroscopy for medical diagnosis. Culinary and hospitality industry publications services (CHIPS), Weimar
80. Romeo MJ, Dukor RK, Diem M (2008) Introduction to spectral imaging, and applications to diagnosis of lymph nodes. In: Diem M, Griffiths PR, Chalmers JM (eds) Vibrational spectroscopy for medical diagnostics, Wiley-VCH Verlag GmbH, Weinheim
81. Lasch P, Beekes M, Schmitt J, Naumann D (2007) Detection of preclinical scrapie from serum by infrared spectroscopy and chemometrics. Anal Bioanal Chem 387:1791–1800
82. Huh SY, Chung AJ, Erickson D (2009) Surface enhanced Raman spectroscopy and its application to molecular and cellular analysis. Microfluid Nanofluid 6:285–297
83. Sikirzhytski V, Virkler K, Lednev IK (2010) Discriminant analysis of Raman spectra for body fluid identification for forensic purposes. Sensors 10:2869–2884