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Metabolomic analysis with ^{1}H-NMR for non-invasive diagnosis of hepatic fibrosis degree in patients with chronic hepatitis C

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ABSTRACT

Background: The assessment of fibrosis degree in liver diseases is based on several non-invasive techniques, but none has been accurate.
Aim: This study employed proton nuclear magnetic resonance spectroscopy to identify metabolic profiles in serum and urine, specific for different fibrosis degree in chronic hepatitis C patients.
Method: 71 plasma, 73 serum, and 578 urine samples were collected. All samples were analyzed using ^{1}H-NMR spectroscopy technique and three different NMR spectra were acquired for each serum/plasma sample. The data analyses were performed by partial least square regression, principal component analysis, and Monte Carlo cross-validation in a supervised methodology.
Results: The cross-validation test correctly assigned each sample to its specific donor with 98.44% accuracy for urine samples and 65% for serum/plasma samples. Advanced fibrosis and cirrhosis were recognized with 71% sensitivity for CPMG plasma spectra and 69% specificity for NOESY serum spectra. Accuracy for NOESY serum spectra was 68%. Noesy spectra recognized advanced fibrosis and cirrhosis with 71% sensitivity, 30% specificity, and 50% accuracy in urine samples.
Conclusion: Metabolomic analysis of urine spectra using ^{1}H-NMR spectroscopy can recognize a specific individual profile in all patients with chronic hepatitis C. However, this method cannot yet differentiate with sufficient accuracy, patients with advanced fibrosis from patients with milder disease.

1. Introduction

The ability to verify the possible deterioration of chronic hepatitis, and thus development of liver fibrosis, is still largely related to the assessment of clinical and instrumental exams with a mediocre diagnostic power in terms of sensitivity and specificity. So far, the search for serum markers or non-invasive imaging techniques, predictors of liver fibrosis degree, has not led to the development of accurate tests. The metabolomic approach with Nuclear Magnetic Resonance (NMR) spectroscopy, capable of identifying the full spectrum of metabolites in the urine and in the peripheral blood samples of subjects [1], can provide a useful solution to achieve a non-invasive diagnosis of fibrosis, or may perhaps allow the identification of metabolites markers of hepatic fibrosis that may possibly be exploited for more simple biochemical tests [2–5]. The aim of this study was to identify the metabolic profiles, in serum and urine samples, related to different stages of hepatic fibrosis in patients with chronic hepatitis C infection (HCV) complicated by liver fibrosis.

2. Materials and methods

Thirty-three consecutive patients with chronic HCV infection and those undergoing different stages of hepatic fibrosis were enrolled: 10 patients were affected by liver cirrhosis (Child-Pugh score A), 23 patients were affected by chronic active hepatitis with a mild or moderate hepatic fibrosis. Informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008). The study was approved by the ethical committee of the Gastroenterology Unit. Hepatic fibrosis was

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investigated using liver biopsy (grading and staging) or a fibroscan mean value >13.5 kPa on at least 10 observations. Patients with any pathological condition that might influence the metabolic profile of the patients (HIV and/or HBV co-infection, pregnancy and lactation, active liver autoimmune diseases, thyroid disorders, malignant neoplasia, renal disease, diabetes mellitus, alcohol consumption >50 g/die or drugs consumption) were excluded from the study. The study was conducted over a period of 3 months, during which patients had to run 3 samples of serum/plasma (time 0, time 1 and time 2), distributed according to a time interval of 30 days. A number of urine samples (between 12 and 24) within 3 months were also obtained, with weekly or biweekly frequency. The patient also had to provide a detailed diet diary on the day prior to each collection of serum/plasma or urine. The urine samples were collected in collection tubes with screw cap Falcon, 15 ml. The plasma was collected in EDTA K2 tubes for 2–6 ml, serum in siliconized tubes 2–6 ml. All urine, plasma, and serum tubes were stored at 4 °C before transfer to the laboratory. Samples of fresh serum-plasma were centrifuged at 3500 rpm for 10 min and RT 3 1000 μl aliquots of each plasma and serum sample were prepared in cryovials and stored at −80 °C (after indication of the code, patient name and date of analysis). The fresh urine samples were divided into five, 1800 μl aliquots for each sample, and then prepared in cryovials and stored at −80 °C. Each stored cryovial was categorized according to a specific alphanumeric coding system. For each blood sample, some biochemical parameters were measured to identify the possible development of metabolic abnormalities which might significantly alter the results of the spectrometer during the study period. The serum/plasma sample was first thawed at room temperature and then 300 μl were transferred to 300 Eppendorf tubes and mixed by inversion before the preparation for NMR analysis. Each sample was diluted to 1:1 with sodium phosphate buffer (70 mM Na2HPO4, 20% (v/v) 2H2O, 0.025% (w/v) NaN 3, 0.8% (TSP), pH 7.4) and then 180 ml of this mixture were placed in NMR tubes of 2.50 mm (Bruker BioSpin Ltd.). In turn, it was inserted into 5 mm Teflon tubing (New Era) and into another 5-mm diameter outer glass tube (New Era). The homogeneity of the magnetic field was previously evaluated. The urine samples were thawed at room temperature and mixed by inversion. Of each sample, 630 μl were centrifuged at a speed of 14,000 rpm for 5 min at 4 °C to pellet the sediment present. Subsequently, 540 μl of supernatant were recovered and added to 60 μl of sodium phosphate buffer (0.2 M Na2HPO4, 0.2 M NaH2PO4 in 100% 2H2O, pH 7.0), containing sodium trimethylsilyl [2,2,3,3-2H4] propionate (TSP) 10 mM, to reference the NMR spectrum to zero once acquired, and 30 mM NaN 3. 450 μl of this mixture was placed in NMR tubes of 4.25 mm (Bruker BioSpin Ltd.) and then analyzed. All Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectra were acquired using a Bruker BioSpin 600.13 MHz spectrometer with a 5 mm diameter cryo-probe CPTCI 1H-13C/31P-2H with coil for the gradient along the Z axis, automatic tuning-matching (ATM) and automatic sampler. A thermocouple PT 100 allows the stabilization of the sample temperature with an accuracy of 0.1 K. Each measurement samples are left for three minutes inside the magnet in order to allow the stabilization of the temperature (300.0 K–310.0 K for the urine and serum and plasma). For each urine sample, one-dimensional spectra (1-D) with water signal suppression using a standard pulse sequence (NOESYpresat, Bruker), were acquired with 64 scans, 64.000 points and a 12.019 Hz spectral width, an acquisition time of 2.7 s, a relaxation delay of 4 s, and a mixing time of 100 ms. NOESY presat, Carr-Purcell-Meiboom-Gill (CPMG, Bruker) spectra and modified diffusion sequences using a diffusion time of 120 ms were acquired for each serum and plasma sample. In this case, NOESYpresat spectra consisted of 64 scans, 98 k data points, a spectral width of 18,028 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s, and a mixing time of 100 ms. CPMG spectra consisted of 64 scans, 74 k data points, a spectral width of 12,019 Hz, an acquisition time of 3.1 s, a relaxation delay of 4 s, and a mixing time of 100 ms. In order to clarify which data can be derived from using a particular spectrum rather than another, it should be stressed that in a NOESY spectrum of serum/plasma samples both signals from small and large metabolites can be seen; in a CPMG spectra, signals from low molecular weight metabolites are predominant (albumin signals are cut off by playing on the different relaxation time as well as those related to lipoproteins, which are very small). For diffusion spectra, signals from low molecular metabolites are eliminated in favor of large molecular metabolites. In this case, diversity of diffusion gradients between large and small molecules is used.

To process spectra, the FID (Free Induction Decays) of each experiment has been multiplied by an exponential function before application of the Fourier transform. After the transformation of the spectra using a correction automatic program implemented in TopSpin (version 2.1, Bruker), the distortion of the baseline phase was corrected. The calibration was performed on the signal of TMSP calibrated on 0.00 ppm. A recalibration on lactate signal placed at 1333 ppm was performed for serum and plasma samples. This is because the TMSP in blood derivatives, in which albumin is present, tends to shift because of albumin binding. The portion of each spectrum, ranged between 0.02 ppm and 10.00 ppm, was segmented into buckets of width of 0.02 ppm and the corresponding spectral region was integrated using AMIX software (Bruker BioSpin). The region of the spectra of urine between 6.0 and 4.5 ppm containing the residual water signal and the signal for urea has been eliminated. For serum and plasma, due to lower efficiency in the water signal suppression, a region between 6.0 and 4.2 ppm was removed. The total area of the spectrum has been calculated on the remaining buckets that were normalized before the statistical analysis [6].

3. Statistical analysis

The regression PLS (Partial Least Squares) was applied to the data matrix and the components that help to explain 99% of the variance have been subjected to an additional CA (Canonical Analysis), to further reduce the number of variables.

This type of statistical model results from informed statistical technique. The information on membership was used on the group of patients with hepatitis C or HCV related cirrhosis. To validate this model, a double cross-validation scheme was used. The original matrix of data was divided into a training set (90% of samples) and a test set (the remaining 10%); while the statistical model was built using the PLS-AC on the training set. Samples belonging to the test set have been used as SVM method (Support Vector Machines). The entire procedure was repeated 100 times, as required by cross-validation scheme of Monte Carlo. To avoid possible bias due to the presence in the test set and training set of samples from the same patient, the validation scheme was built so that the set of samples of serum, plasma or urine, as appropriate, of the same patient was present in every step of the validation; only in the training set or in the test set, never in both.

The sensitivity was calculated by the average recognition values obtained from each sample belonging to patients diagnosed as healthy. Specificity was calculated by the average recognition values obtained from each sample belonging to patients diagnosed as sick. The accuracy was calculated by averaging recognition values obtained from each sample. The test performance values were represented in a confusion matrix.

4. Results

In order to verify the accuracy in the identification of specific metabolic profiles, the system's ability to identify a specific metabolic profile for each patient from multiple samples of urine,

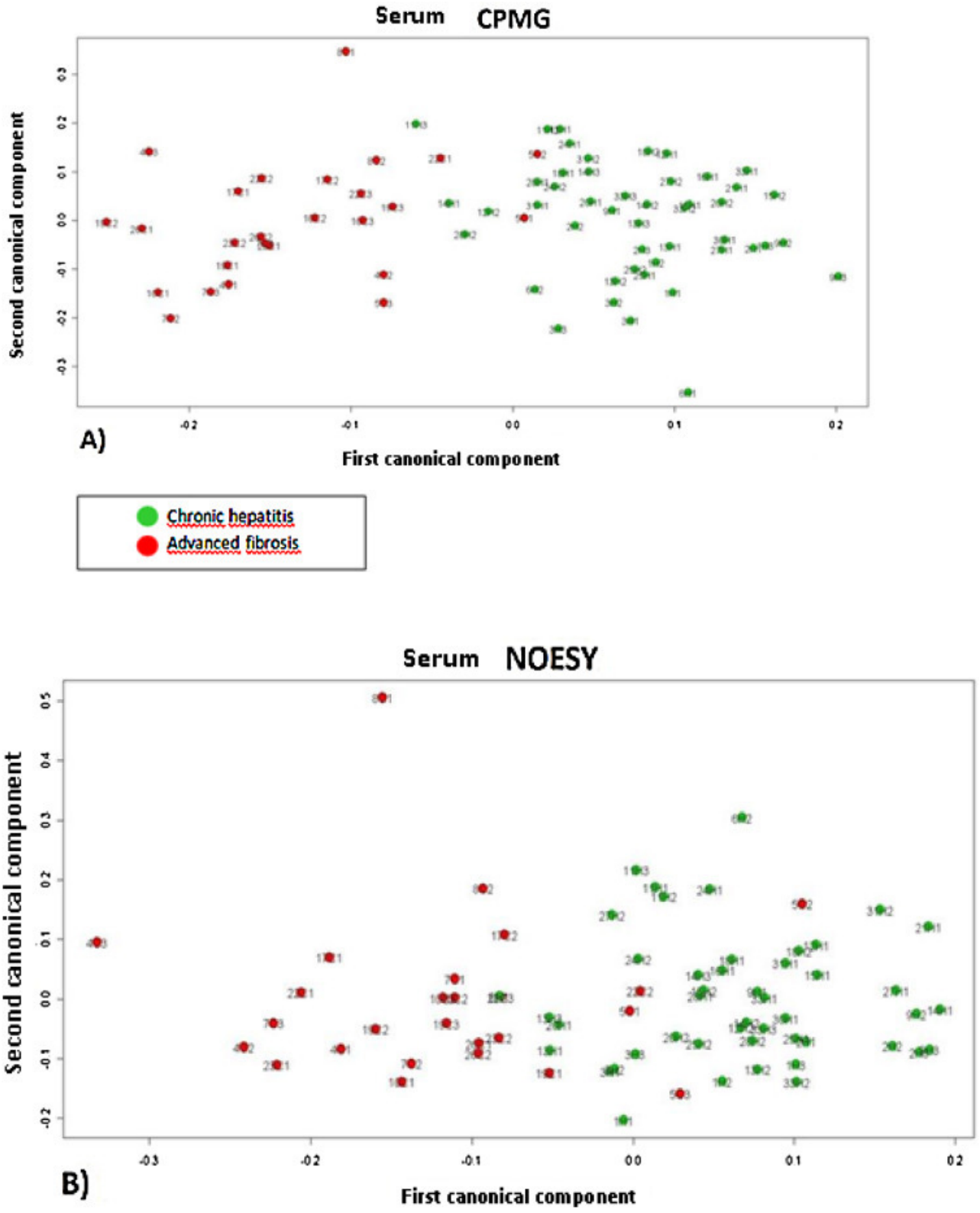

Fig. 1. (A) Plot of the first two canonical components of the PLS-CA relative to the spectrum of serum CPMG. (B) Plot of the first two canonical components of the PLS-CA relative to the spectrum of serum NOESY.

serum and plasma was analyzed. The statistical model constructed by analysis PLS-CA had an excellent clustering of urine samples. Only a few spectra were misclassified by the system. Overall, the method showed an accuracy of 98.44% in the assignment of the individual patient urine samples showing that with 8–20 urine samples, it is possible to obtain the metabolic profile of a specific patient. This high accuracy implied the validation of the method of analysis used to discriminate the metabolic profile and to assign a specific metabolic profile to each patient. The analysis for the recognition of the patient described above for the urine performed on multiple

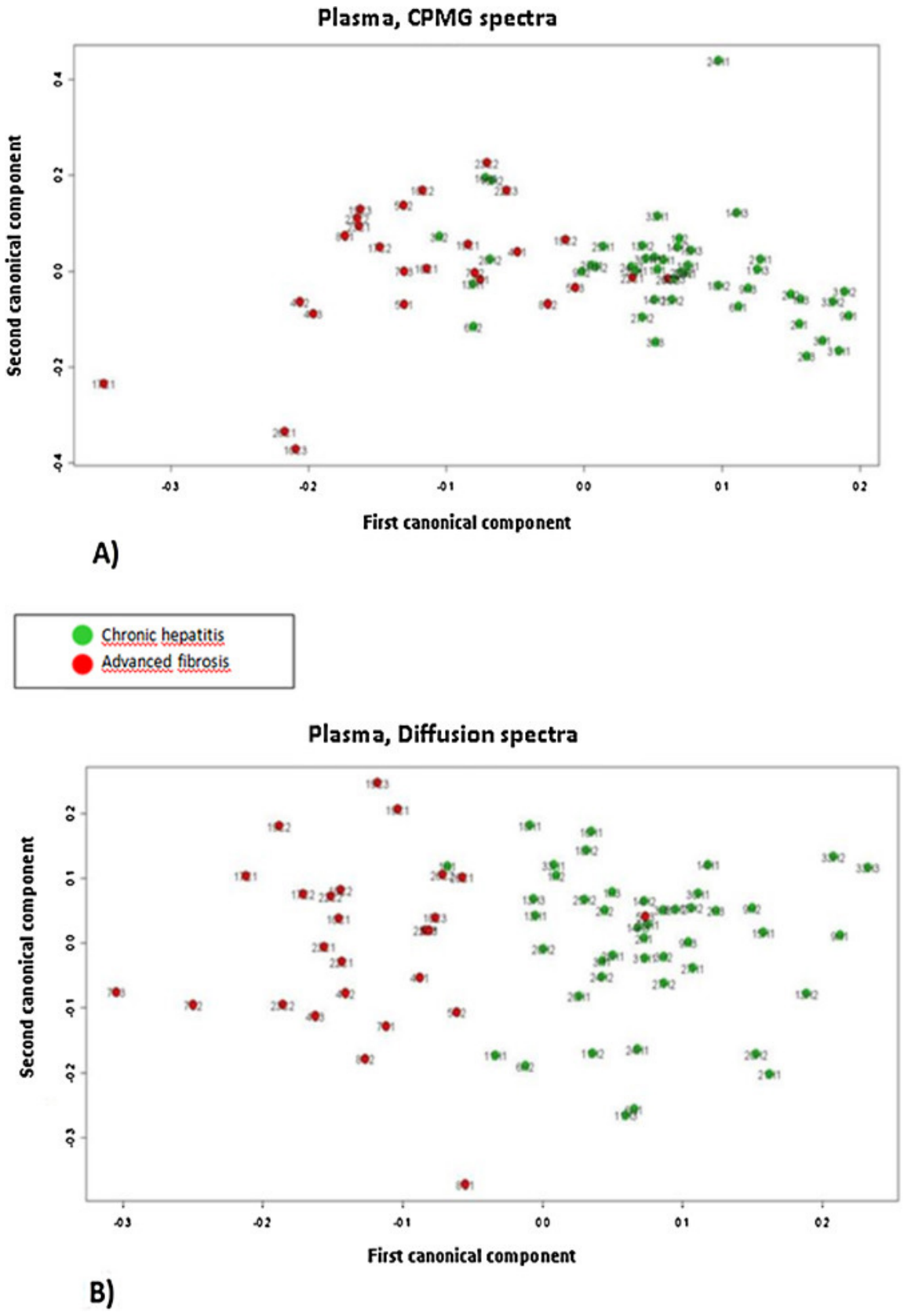

Fig. 2. Plot of the two components of the canonical PLS-CA relating to CPMG spectra (A) and Diffusion (B) plasma.

samples of serum and plasma, gave less accurate results. The accuracy was in fact 50% for patient with only two samples of serum and plasma, and 65% for patients with three samples of serum and plasma. When validation of the method was done, the statistical model on samples of urine, serum, and plasma was applied to eval-

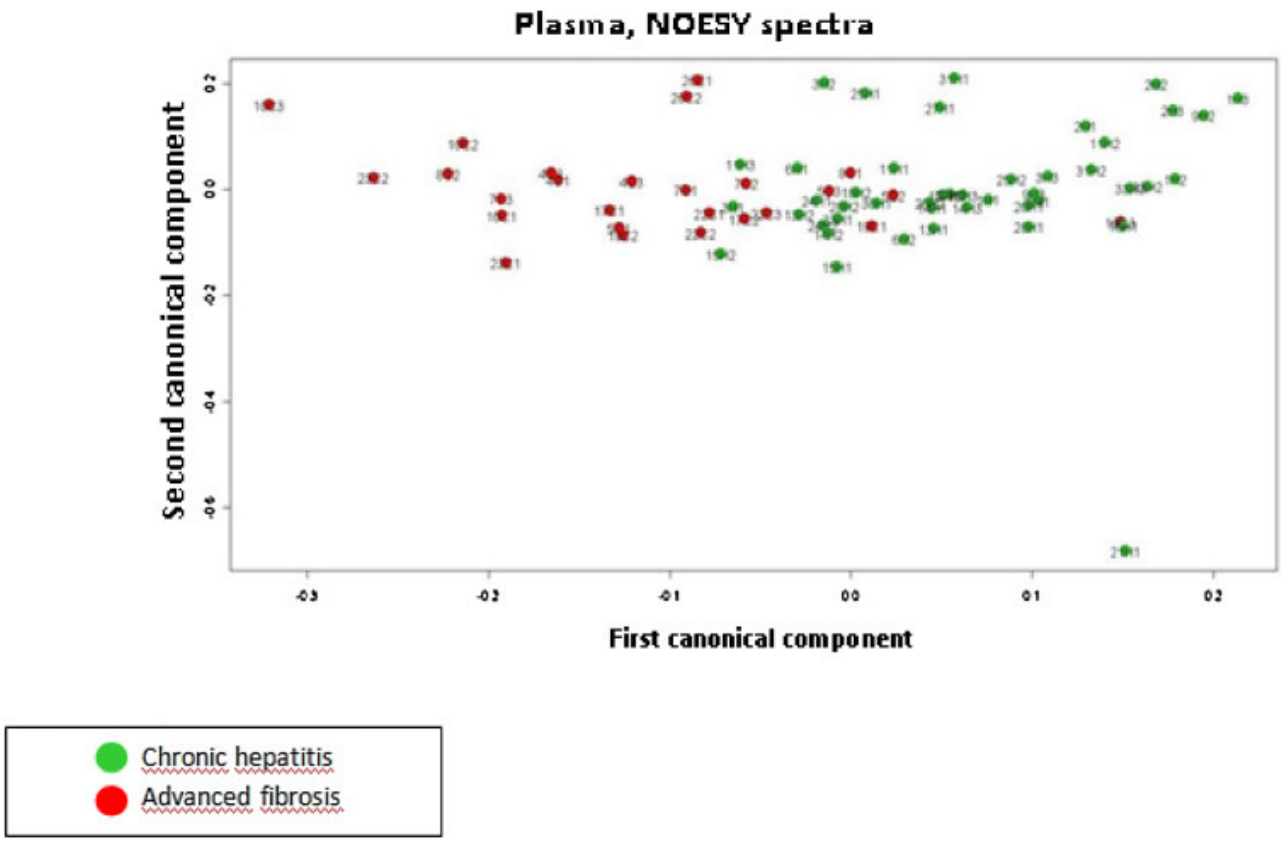

Fig. 3. Plot of the first two canonical components of the PLS-CA relative to the spectrum of plasma NOESY.

uate the possibility of distinguishing patients with liver cirrhosis from those with only hepatitis.

The analysis of sera for the CPMG spectrum according to the diagnosis of hepatitis or cirrhosis showed a good clustering of the samples belonging to the two categories of patients. Only a limited number of out layers were in the plot (Fig. 1A). The analysis of the NOESY spectrum showed a lower accuracy in clustering the samples from different groups, with a significantly higher number of samples misclassified (Fig. 1B). The analysis of the spectra with CPMG, Diffusion, and NOESY method, on plasma samples showed good clustering of data in the two groups of patients. However, in this case there was evidence of numerous outliers with overlapping of the two groups of patients. This phenomenon appeared to be less apparent with diffusion analysis, in which the signal of micromolecules is deleted (Figs. 2A and B and 3).

The analysis of urine samples, where the concentration of metabolites is much lower compared to the blood samples, was performed on the full spectrum since the signal suppression of certain classes of molecules reduced the ability of the discriminative test drastically. The analysis technique with NOESY showed an extensive overlap of the spectra of the samples from the two groups of patients, with a good polarization on both sides due to the large number of samples (Fig. 4). The method of analysis performed by calculating the percentage of times that the system correctly recognized the sample as belonging to the respective group of patients, showed rather unsatisfactory values. In all, the values of recognition for cirrhotic patients are quite low, in fact only two of 10 patients had a high degree of recognition, not less than 80%. For at least 4 patients, a high variability was demonstrated on recognition of individual sample close to 90% for some samples and 0% for some other samples obtained from the same patient.

The data concerning the recognition of samples of patients with chronic hepatitis were better obtained. The recognition value per patient was never below 50% and more than half of the cases showed a value close to 90%. The sensitivity for both CPMG and NOESY spectrum of the serum was approximately 60%. The specificity was limited to 50% for the CPMG spectrum and 69% for the NOESY spectrum. The accuracy of the test on serum has amounted to 56% for the CPMG spectrum and 48% for the NOESY spectrum. The analysis of plasma samples showed sensitivity of 71% and a specificity of 50% for the CPMG spectrum. The Diffusion spectrum provided a sensitivity of 42% and a specificity of 67%. The NOESY spectrum showed sensitivity of 66% and a specificity of 52%. The overall accuracy of analysis on plasma samples were 50% for the CPMG spectrum, 67% for the Diffusion spectrum, and 52% for NOESY spectrum. The performance characteristics of test on urine samples showed a sensitivity of 71% and a specificity of 30% with an overall accuracy of 50% (Table 1 A-B-C-D).

5. Discussion

This study represented the first application of high-field ^{1}H NMR metabolomics for detection of a specific metabolic profile, which allow differentiation between mild/moderate fibrosis and cirrhosis. The data of this study suggested that the metabolomics analysis with ^{1}H-NMR spectroscopy can be applied to study viral hepatitis, obtaining with a considerable accuracy a urinary metabolomic profile fingerprint. As expected, the accuracy was lower in serum and plasma samples due to fewer numbers of samples available. However, the data suggested that the distinction between non-cirrhotic hepatitis and cirrhosis is not very accurate with this system and that the clustering observed was due to the tendency to cluster samples belonging to the same patient. The lowest discriminative power in terms of specificity has been achieved with the NOESY spectrum of the urine. The high variability of daily urinary metabolites related to diet had a negative impact on the performance of the test, while

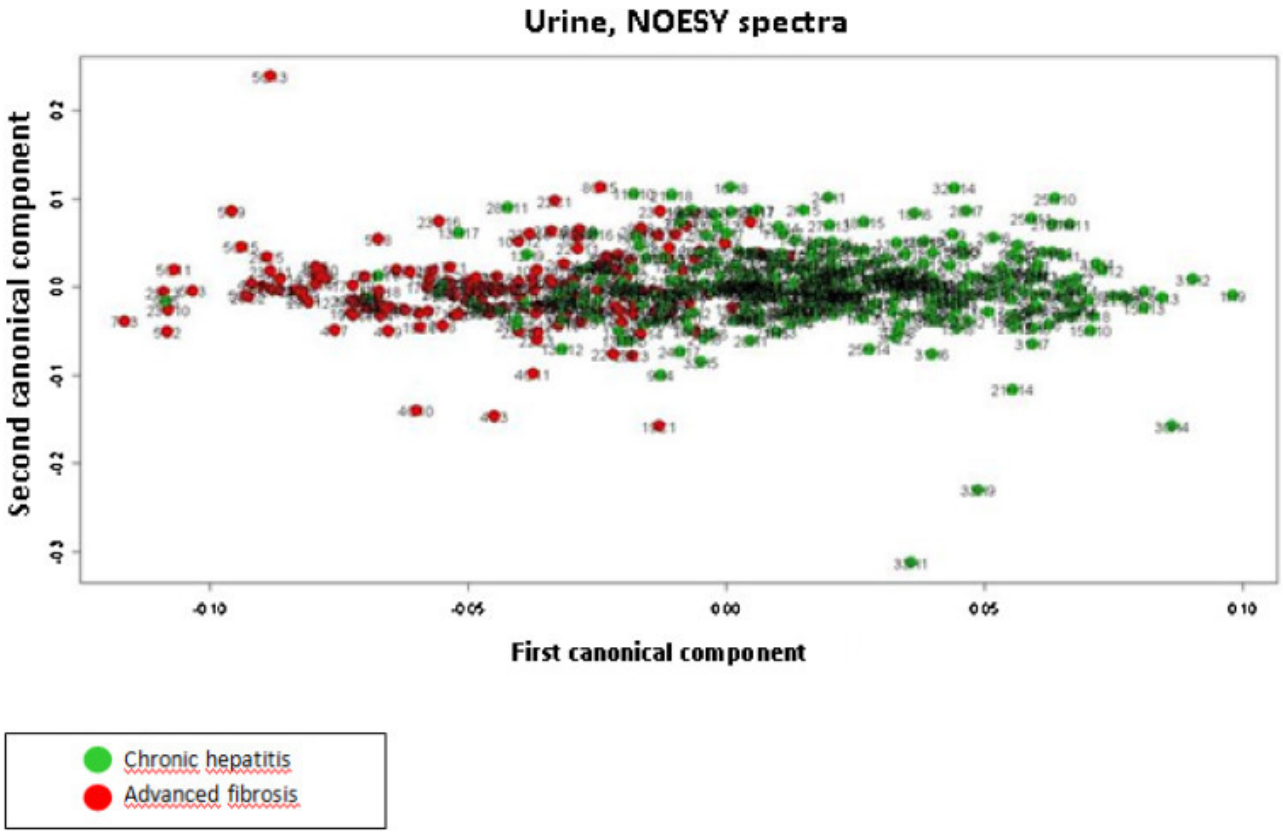

Fig. 4. Plot of the first two canonical components of the PLS-CA relating to NOESY spectra of urine.

Table 1
Indices of test performance for each analyzed spectrum.

Serum, CPMG	Hepatitis	Cirrhosis	31 patients (21vs10) 73 samples (47vs26)
Hepatitis	0.59	0.41	Sensibility
Cirrhosis	0.47	0.53	Specificity
			Accuracy =0.56

Serum, NOESY	Hepatitis	Cirrhosis	31 patients (21vs10) 73 samples (47vs26)
Hepatitis	0.60	0.40	Sensibility
Cirrhosis	0.31	0.69	Specificity
			Accuracy =0.48

Plasma, CPMG	Hepatitis	Cirrhosis	30 patients (20vs10) 71 samples (45vs26)
Hepatitis	0.71	0.29	Sensibility
Cirrhosis	0.50	0.50	Specificity
			Accuracy =0.62

Plasma, Diffusion	Hepatitis	Cirrhosis	30 patients (20vs10) 71 samples (45vs26)
Hepatitis	0.42	0.58	Sensibility
Cirrhosis	0.33	0.67	Specificity
			Accuracy =0.47

Plasma, NOESY	Hepatitis	Cirrhosis	30 patients (20vs10) 71 samples (45vs26)
Hepatitis	0.66	0.34	Sensibility
Cirrhosis	0.48	0.52	Specificity
			Accuracy =0.59

Urine, NOESY	Hepatitis	Cirrhosis	33 patients (22vs11) 578 samples (398vs180)
Hepatitis	0.71	0.29	Sensibility
Cirrhosis	0.70	0.30	Specificity
			Accuracy =0.50

the spectrum of the urine provided the highest value of sensitivity of the test. The expectation of greatest dispersion of data related to a large number of urine samples analyzed was not confirmed; instead the test showed a good ability to discriminate patients by analysis of urine. This suggested that it was useful to investigate the urinary metabolites that allowed this clusterization. According to the diagnosis, the diffusion spectrum of plasma samples with suppression of signal of macromolecules and the NOESY spectrum of serum provided better specificity value for distinguishing the samples, although still unsatisfactory. The NOESY spectrum analysis of serum samples, however, showed a poor sensitivity when compared to the spectrum of the CPMG plasma which showed a sensitivity value of 71%. This observation was probably due to loss of data relating to micromolecules due to the phenomenon of superposition of the curves caused by the analysis of the complete spectrum. According to literature [7,8], the study showed the fundamental role of small molecules in capturing the subtle changes that affect the metabolism of different subjects and pathological conditions. An accuracy of 62% was obtained on CPMG spectra of plasma. Performance indices of our test indicated a low specificity. It was possible that this limit was influenced by the relatively low size in our series. The metabolomic examination performed with different methods on different biological substrates was unable to distinguish non-cirrhotic patients from cirrhotic patients with sufficient accuracy. It is possible that this inability depends on the limited variation of the metabolic profile associated with initial cirrhosis, compared to chronic hepatitis with mild fibrosis. It is well known that these two groups of patients differ only on the basis of the histopathological findings, demonstrating the presence of regenerative nodules in the liver, whilst other indices of function and hepatocellular damage are substantially identical. Cirrhotic patients with advanced disease, in which the functional changes of the liver are more marked, were excluded. This contributed to the difficulty to differentiating the two groups. The findings from the tests are quite similar with those reported in the literature for other non-invasive tests based on direct and indirect biochemical parameters, whose ability to differentiate initial cirrhosis compared to a moderate or advanced fibrosis is very limited [9].

The data are in disagreement with those reported in a study of guinea pigs, in which hepatitis and cirrhosis were induced acutely (within about 4 months) through the administration of thioacetamide [10]. In this study, the method of NMR spectroscopy applied on samples of mouse plasma, showed a high discriminative potential between different stages of disease. However, it is questionable whether this experimental model of animal cirrhosis really reflects what happens in human disease in which the evolution to cirrhosis develops over a period of decades and not in a period of few weeks after an acute injury. A recent study showed that the ^{1}H-NMR metabolomics technique demonstrated excellent discriminative ability in comparing groups of healthy subjects and patients with cirrhosis and HCC [11]. Unfortunately, the results of this study are not directly comparable with our views of the different characteristics of the cases considered. Nevertheless, they suggest the existence of a metabolic profile characteristic of cirrhotic patients; while in this study, cirrhotic patients were not sufficiently differentiated from those with hepatitis. This fact is not a surprise considering that the transition from chronic hepatitis to cirrhosis occurs along a continuum of metabolic changes that they are clearly distinguishable at their ends, but not at all intermediate stages.

6. Conclusion

In conclusion, this study demonstrated that the technique of metabolomics analysis by ^{1}H-NMR spectroscopy is a powerful tool for determining the specific metabolic spectrum of a subject mainly from urine samples. The method manages to identify an individual fingerprint, despite the daily variability of physiological metabolism. However, the method has not achieved sufficient diagnostic accuracy to differentiate patients with chronic HCV from patients with compensated cirrhosis. It remains an open question as to whether this incapacity is linked to the objective difficulty of differentiating two disease states linked by a slow and continuous progression, or to the limits of the methodology in capturing the subtle variations of metabolic structure associated with these conditions.

Conflict of interest

None declared.

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