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Nuclear magnetic resonance spectroscopic analysis of salivary metabolome in sarcoidosis

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Abstract. *Background:* Sarcoidosis is a systemic granulomatous disease of unknown cause which has diverse clinical impacts, ranging from benign to very severe, which may therefore require systemic treatment. Only a few tools are currently available to monitor management in these patients. *Objectives:* As sarcoidosis is known to affect salivary glands, we hypothesized that analysis of saliva could reveal valuable biomarkers for disease management. We designed a comparative analysis of salivary metabolomics in patients and controls using Nuclear Magnetic Resonance (NMR). *Methods:* Metabolomic profiles of saliva collected from 24 sarcoidosis patients and 45 controls were obtained by proton NMR spectroscopy with multivariate statistical analysis, followed by metabolite identification and pathway analysis. Oral and dental examinations were performed concomitantly, together with assessment of smoking habits. *Results:* A predictive salivary metabolomic signature associated with sarcoidosis was computed with the Orthogonal Partial least squares discriminant analysis (OPLS) model. Six metabolites were altered in samples from sarcoidosis patients compared to controls, including decreased levels of methanol and butyrate and increased levels of lactate, acetate and N-butyrate. *Conclusion:* This study showed that NMR metabolomics can discriminate saliva samples from sarcoidosis patients and controls. According to these preliminary results, saliva studies in sarcoidosis patients would be particularly useful to identify potentially relevant biomarkers. A study based on a larger number of patients at different stages of the disease or with treated patients is needed to assess the clinical relevance of NMR metabolomics in sarcoidosis. *(Sarcoidosis Vasc Diffuse Lung Dis 2016; 33: 10-16)*

Key words: sarcoidosis, saliva, NMR metabolomics

Introduction

Sarcoidosis is a systemic disease of unknown cause, characterized by the formation of immune non-caseating granulomas (1). Sarcoidosis most commonly involves the respiratory and lymphatic systems, but virtually all organs can be affected. Sarcoidosis may have diverse clinical impacts, ranging from benign to very severe and particularly aggressive

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sarcoidosis needs to be treated, mostly by steroids. However, some patients do not respond to treatment or subsequently relapse (1). Although several laboratory test abnormalities are observed during the course of the disease, no consensus has been reached concerning the recommended laboratory tests to assess disease activity or for follow-up and treatment monitoring (2). ^{18}FdGlucose uptake in patients has been shown to be a very sensitive method for the assessment of inflammatory and granulomatous reactions (3). However, it is expensive and exposes patients to ionizing radiation and therefore cannot be frequently repeated. Recently, chitotriosidase, a biomarker of macrophage activation, has been shown to be of clinical relevance for the follow-up of sarcoidosis granulomatous disease (4). A better knowledge of the metabolism of granulomas is therefore particularly challenging.

The salivary glands (minor or main glands such as the parotid) are known to be involved in sarcoidosis (5), as biopsy of the minor salivary glands, a routine investigation in this clinical setting, reveals granulomatous infiltration in about one half of cases (5). Moreover, in 5 to 10% of cases, several types of clinical expression (hypersalivation or hyposalivation, xerostomia, or more rarely parotiditis) are observed. In contrast with the salivary glands, specific buccal or periodontal sites of the disease are considered to be uncommon (5–7). In this context, we hypothesized that changes in the saliva of patients with sarcoidosis compared to control subjects could be related to granulomatous involvement of the salivary glands. We therefore designed a comparative salivary metabolomic analysis in patients and controls using Nuclear Magnetic Resonance (NMR) spectroscopy to identify specific changes of salivary metabolite content in sarcoidosis, as already demonstrated for other systemic diseases (8,9). We demonstrated that NMR metabolomic analysis of saliva can identify a metabolomic signature of active sarcoidosis.

Methods

Saliva samples from consecutive sarcoidosis patients and control subjects were collected from January 2011 to July 2011 (26 patients with sarcoidosis and a control group of 50 healthy subjects). Sarcoidosis patients were followed or hospitalized in the Avicenne University Hospital Respiratory Medicine Department (93000 Bobigny France). Inclusion criteria were: sarcoidosis meeting the ATS/ERS/WASOG diagnostic criteria (10), the diagnosis had to have been established during the previous 3 years, either clinically active disease (20 patients) or a serum angiotensin converting enzyme level at least twice the upper limit of normal (6 patients). We considered sarcoidosis to be active in the presence of new symptoms or worsening symptoms, deterioration of pulmonary function tests or chest CT-scan, or hypermetabolic state, as shown on ^{18}FDG PET scan (10). A serum angiotensin converting enzyme level at least twice the upper limit of normal was considered to be a marker of a high granuloma burden (11). None of the patients had a history of ongoing or recent systemic corticosteroid or immunosuppressive therapy. As the diagnosis of sarcoidosis had been confirmed in patients enrolled, minor salivary gland biopsy was not considered in this study. Healthy subjects, including visitors and hospital workers, were recruited by means of a poster displayed in the hospital and the diagnosis of sarcoidosis was excluded by a clinician before inclusion. The periodontal status was evaluated in patients and controls by a trained dentist using the following indices: Plaque Control Record (PCR), Gingival Bleeding Index (GBI) and, when periodontitis was detected, Periodontal Pocket Depth (PPD) was measured and Clinical Attachment Loss (CAL) was calculated (12). To avoid confusion, periodontitis was defined as active destruction of the periodontal tissue evidenced by the presence of at least > 2 interproximal sites with both PPD ≥ 3 mm and ≥ 3 mm CAL (not on the same tooth and non-adjacent teeth) (13). An assessment of smoking habits was also recorded. Written informed consent was obtained from all patients prior to their participation. This study received institutional review board approval N° IRB00006477.

Saliva collection

Saliva secretion was stimulated by masticating an amorphous gum (GC France, 94384 Bonneuil-sur-Marne France). A minimum of 5 mL of saliva was collected. Saliva pH and time elapsed since the last meal were recorded. Samples were stored at -20°C before NMR analysis.

NMR analysis

For NMR, 100 µL of deuterium oxide were added to 600 µL of thawed saliva. Proton NMR spectra were acquired at 298 K on a Varian INOVA NMR spectrometer at a proton frequency of 500 MHz with a water signal suppression sequence. Spectra were processed by NMR pipe and binning (0.01 ppm) was performed using R software®. The resulting matrix was analyzed with SIMCA-P+® and MATLAB® with an in-house script.

Statistical analysis

A multivariate model was used, as it reduces the dimensionality of the data to focus on a couple of latent variables (for a review see (14) and (15)). Principal component analysis (PCA) was performed to detect any group separation based on NMR spectra variability and to exclude possible outlier cases. An orthogonal projection to latent-structure (OPLS) analysis was computed to determine the NMR spectra variations between healthy subjects and patients with sarcoidosis. The statistical performances of the model were assessed by R2 (Y), representing the explained variation and Q2 (Y) estimating the model predictability. Q2 (Y) was calculated using a 7-fold cross-validation. The results are presented as a score plot, demonstrating the discrimination between groups, and a loading-plot showing the spectral region responsible for this discrimination. To estimate the ability of the model to correctly classify a new data set, a CV-AUROC (Cross Validated – Area Under Receiver Operating Characteristic) curve was build using the predictions of each cross-validation set (16).

Metabolite identification and metabolic pathway analysis

The discriminating buckets were assigned to metabolites according to their spectral characteristics (chemical shifts and multiplicity, 2D cross-correlation peaks) with reference to previous publications (17,18) and the HMDB website (19). Metabolic pathway analysis was performed using MetPA (http://metpa.metabolomics.ca) (20).

Results

Patients and controls

Characteristics of patients and controls are summarized in Table 1 showing that the sarcoidosis patients and the healthy subjects were not significantly different in terms of smoking habits (43% vs. 30%, p=0.261), while a significant difference was observed in terms of age (mean age 45.9 yr vs. 35.4 yr, p<0.01, respectively). Interestingly, a significantly higher frequency of periodontal disease was observed in the group of sarcoidosis patients compared to controls (18% vs. 6%, p= 0.0037, respectively).

Table 1. Characteristics of patients and controls

	Sarcoidosis	Controls	P value
Number*	24	45	
Age (years)	43.5 +/- 1.9	36.5 +/-2.0	0.006
Gender (men/women)	0.71	0.36	0.114
Sarcoidosis			
Stage I	7		
Stage II	11	Not applicable	
Stages III &IV	6		
Treatment	None	None	
Smoker or ex-smoker	14/24	15/45	0.237
Periodontal evaluation	18/24	45/45	
Periodontitis	8/18	2/45	0.002

* 26 sarcoidosis patients and 50 controls were initially enrolled, but 2 sarcoidosis patients and 5 controls were subsequently excluded from the study

Multivariate analysis of metabolomics data

NMR spectra were obtained for the 76 saliva samples. Seven of these 76 samples were excluded from the analysis (two cases of sarcoidosis and 5 controls) according to the PCA variability analysis. One of the sarcoidosis samples was taken from a treated patient and the lactate concentration was abnormally high in the other sample. The other 5 spectra from saliva collected in controls showed impaired water signal suppression.

The supervised OPLS method was used to discriminate between sarcoidosis and healthy subjects. The score-plot of the model computed is shown in Figure 1, demonstrating a discrimination between the spectra of controls and sarcoidosis patients with

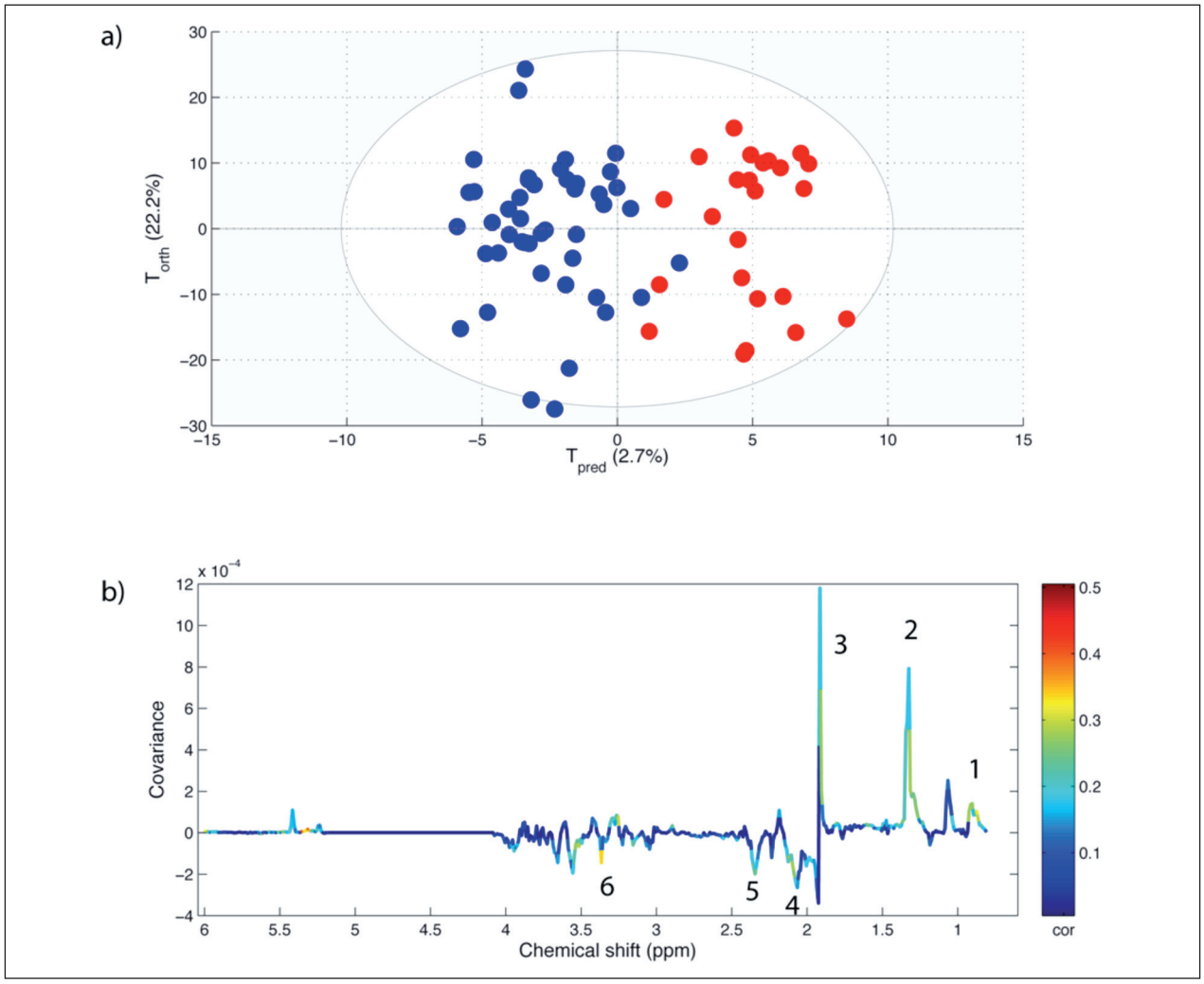

Fig. 1. (a) Orthogonal projection to latent-structure (OPLS) score plot (see material and methods). Each point in the score plot represents the projection of an NMR spectrum (and therefore a patient's saliva sample) on the predictive (horizontal axis) and orthogonal (vertical axis) components of the model. Patients with sarcoidosis are shown in red, while controls are shown in blue. This figure therefore, explains the discrimination between the control and sarcoidosis groups along the predictive axis. (b). Loading plots: for each bucket, the frequency (ppm) is plotted with its intensity and colour label, representing the covariance and the correlation between the NMR signal intensity and group membership, respectively. The covariance represents the "load" of each bucket on the predictive axis of the score plot. Positive covariance corresponds to a metabolite which is present at a higher concentration in patients with sarcoidosis, while negative covariance corresponds to a metabolite present at a higher concentration in controls. Metabolites are considered to be discriminative when their correlation is greater than 0.3 (yellow label). Discriminative metabolites are shown as follows: N-Butyrate (1); Lactate (2); Acetate (3); N AcetylGlycoprotein (4); Butyrate (5); Methanol (6)

R2(Y) = 0.79 and Q2(Y) = 0.42. Overall, the classification performance of this model was evaluated by the area under the ROC (CV-AUROC) of 0.87, demonstrating the quality and robustness of this model (Figure 2). NMR metabolomic results, including spectral identifications and correlation values, obtained with the 71 saliva samples are summarized in Table 2.

Metabolite identification and metabolic pathway analysis

According to the loading plot presented in Figure 1b, the discrimination between controls and sarcoidosis patients was established on the basis of a decrease in methanol and butyrate contents and an increase in lactate, acetate and N-butyrate contents in the saliva of sarcoidosis patients (Table 2). These metabolites were submitted to pathway analysis using MetPA. Two metabolic pathways were significantly ($p<0.05$) modulated in sarcoidosis patients. These 2 intimately related pathways are associated with glycolysis-gluconeogenesis and pyruvate metabolism, each of those involving acetate and lactate.

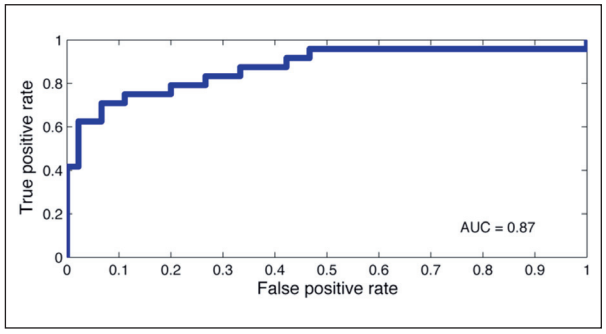

Fig. 2. CV-AUROC (Cross Validated – Area Under Receiver Operating Characteristic) curve, based on classification of sarcoidosis and control spectra during the cross-validation process. The ROC curve is based on the calculation of true- and false-positive rates

Table 2. Metabolite identification and correlation with sarcoidosis

Metabolite	Ppm	Correlation
N-Butyrate(1)	0.9	+ 0.32
Lactate (2)	1.33	+0.3
Acetate (3)	1.92	+0.3
N AcetylGlycoprotein (4)	2.01	- 0.25
Butyrate (5)	2.35	-0.25
Methanol (6)	3.37	-0.34

Discussion

In this pilot study, we compared the metabolomic profiles of saliva collected from patients with sarcoidosis and healthy controls. Metabolomic analysis showed that salivary metabolite concentrations were significantly different between these two groups. As saliva is a biofluid partially composed of fluid secreted by the salivary glands and elements derived from plasma, saliva analysis may therefore be used to investigate both local and systemic phenomena (21).

To our knowledge, this is the first study using NMR to investigate saliva in sarcoidosis. Nevertheless, this method has been successfully used for the study of biological fluids in various diseases including cancer (22) or infectious diseases (23), as saliva is an easily accessible biofluid with a high potential for NMR analysis (17,24).

Our computed OPLS model, based on metabolite concentration variations, shows that only few metabolites, particularly butyrate and lactate, are modulated in sarcoidosis compared to controls. However, the statistical parameters obtained, including the CV-AUROC, demonstrate the reliability of the model and even its potential predictability. Various hypotheses can be formulated to explain the differences between the two groups. First, sarcoidosis is an inflammatory granulomatous disease with modification of glucose metabolism, as suggested by ^{18}FdG uptake (25). Glucose hyper-metabolism is also evidenced by the results of this study, as the observed increase of lactate levels can be considered as a marker of increased anaerobic glycolysis. Metabolic pathway analysis suggested an increase in anaerobic metabolism in sarcoidosis patients compared to controls. We can hypothesise a potential role of hypoxia in granulomas (26), as already described in other granulomatous diseases (27). Bacterial colonization may also be modified in the oral cavity of patients with sarcoidosis. Butyric acid is a known product of *P. gingivalis* and *F. nucleatum*, which cause periodontal disease (28–30). The oral bacterial context in sarcoidosis therefore needs to be studied in more detail. The increased methanol content may also be the result of microbial metabolism.

Several characteristics of our study should be underlined. Different factors, in particular age and periodontal status were considered as supervising

factors in OPLS analysis and none of them was able to discriminate subjects grouped according to each of these factors.

This analysis of potential causes of variability suggests that the variability due to the presence of sarcoidosis is significantly greater than the other sources of variability. The sample size was limited to 69 subjects in the present study including 24 sarcoidosis patients. Nevertheless, saliva from sarcoidosis patients was able to be discriminated from saliva from the 45 controls. The number of sarcoidosis cases of this study can be considered to be relevant in view of the prevalence of this disease. Nevertheless, despite the limited sample size and age dispersion, saliva samples from control subjects and sarcoidosis patients could be discriminated with a statistically significant model.

Another interesting finding of this study is the higher frequency of periodontal disease observed in patients with sarcoidosis compared to controls. No significant difference in smoking habits, known to be a cause of periodontal disease, was observed between the two groups. This higher frequency of periodontal disease could be due to the age difference, but it is more likely due to the fact that periodontitis is frequently associated with various systemic inflammatory diseases (31). It can therefore be hypothesized that the real prevalence of periodontal disease in sarcoidosis may be underestimated and deserves to be specifically evaluated.

In conclusion, a metabolomic profile indicating the presence of sarcoidosis was identified in saliva. These results are in line with those of other non-invasive approaches such as exhaled breath condensate analysis, which also appears to be promising (32). This preliminary study shows that this new non-invasive method based on NMR analysis of saliva could be of potential clinical relevance in sarcoidosis. Moreover, analysis of metabolomic signatures contributes to a better understanding of the disease and the possible identification of biomarkers of the disease activity before and during treatment. However, the findings of this exploratory study now need to be confirmed by an independent study on a larger cohort of patients and controls including other biomarkers, which have recently been shown to be of potential interest. Moreover in such a larger study, metabolomic analysis should be performed concomitantly on other biofluids such as serum or urine.

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