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Short communication

Applying biofluid ^{1}H NMR-based metabonomic techniques to distinguish between HIV-1 positive/AIDS patients on antiretroviral treatment and HIV-1 negative individuals

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Abstract

Metabonomics, the study of metabolites and their role in various physiological states, is a novel methodology arising from the post-genomics era and has extensive biomedical application. This technology has exhibited vast success in the identification and study of human diseases and may find further application in the study of HIV/AIDS. Specifically, the wide range of clinical and metabolic abnormalities associated with the use of antiretroviral (ARV) treatment may be investigated. To this end, this preliminary study evaluated whether metabonomic techniques could distinguish between HIV-1 positive/AIDS patients utilizing antiretroviral therapy and HIV-1 negative individuals. Serum metabolic profiles determined by ^{1}H nuclear magnetic resonance (NMR) spectroscopy combined with pattern recognition analysis of the data showed that this distinction was attainable; suggesting that ARV-associated side-effects could be monitored utilizing NMR metabonomic techniques.

Keywords: Metabonomics; HIV/AIDS; Antiretroviral therapy; ^{1}H NMR spectroscopy; Metabolic profiling

1. Introduction

^{1}H NMR spectroscopy of biofluids (urine, serum/plasma) and tissue generates comprehensive biochemical profiles of low molecular weight endogenous metabolites [1]. The NMR spectral profiles resulting from this analysis are, however, complex and more easily interpreted with automated data reduction and chemometric analysis [2] such as principal components analysis (PCA). As to date, ^{1}H NMR spectroscopy-based metabonomics have been predominantly applied to animal studies with information elucidated on gender, strain of animal [3,4], diurnal variation, response to toxins [4] and disease. Animal studies benefit from minimal inherent variability between subjects because factors such as diet, age, gender and other environmental influences are under the control of the investigator [5]. Although greater variability exists in the human population, studies on urine and plasma samples from healthy human volunteers showed that plasma spectra exhibited relatively low inter- and intra-individual variability between subjects and study days [5], suggesting that consistent clinical metabonomic studies could be conducted. As such, metabonomics have been applied to both diseased and healthy [6] human states and has found particular application in the investigation of toxicological mechanisms, disease diagnosis and response to therapy. Notable metabonomic applications include the distinction between type 2 diabetic patients and healthy volunteers [7], diagnosis of coronary heart disease [8] and investigation of hypertension [9]. The potential applications of metabonomics to the identification and study of human disease is vast. ^{1}H NMR spectroscopy-based metabonomics could find extensive application in the monitoring of the biological side-effects of antiretrovirals used for the therapeutic treatment of HIV/AIDS. A wide range of clinical and metabolic complications are associated with the use of anti-retroviral treatment; including lipodystropy syndromes (characterized by either localized fat loss in the face or limbs or fat accumulation in the abdominal cavity, breasts or dorso–cervical region), hyperlipidaemia, insulin resistance and hyperglycemia (reviewed in Ref. [10]). Metabolic disorders of HIV positive/AIDS patients

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are monitored closely as they may lead to an increased risk of cardiovascular disease and type 2 diabetes mellitus and subsequently death [11,12]. Techniques used to assess fat distribution and body composition in HIV positive/AIDS patients include dual-energy X-ray absorptiometry (DXA), computed tomography (CT) and magnetic resonance imaging (MRI). Glucose and insulin irregularities are measured by radioimmunoassay. In addition to the ease of use and simple sample preparation, ^{1}H NMR spectroscopy may potentially monitor and correlate metabolic disorders and contribute knowledge to the study of the adverse side-effects caused by antiretroviral therapy. To this end, this preliminary study demonstrated that distinction could be made between HIV-1 positive/AIDS patients utilizing antiretroviral therapy and HIV-1 negative individuals based on ^{1}H NMR metabolic profiles. Samples from HIV/AIDS patients not using antiretrovirals were included to illustrate that this distinction was based on the use of ARV's.

2. Materials and methods

2.1. Sample collection

Blood was drawn from 34 HIV-1 positive patients attending the HIV/AIDS clinic at the Helen Joseph Hospital in Johannesburg, South Africa. At the time of collection, 29 patients were undergoing treatment with combination antiretroviral therapy while five patients had not previously utilized antiretroviral agents. Samples were allowed to clot in non-anti-coagulant vacutainers (ProfMedical, RSA) for 2 h at room temperature (RT) and serum was separated after centrifugation at $1028 \times g$ and RT. Aliquots of serum were stored at $-80\,^{\circ}$C until assayed. HIV status and use of anti-retrovirals by each patient was determined by hospital records and where available, viral load quantities as determined by nucleic acid sequence-based amplification (NASBA) polymerase chain reaction (PCR) were obtained. In the same manner HIV negative serum was collected from 29 HIV-1 negative laboratory personnel (status confirmed with HIV-1/2 Determine^{TM} rapid tests, Abbott Laboratories, Abbott Park, IL) with no known current metabolic or other medical condition. Informed consent was obtained from all volunteers and patient anonymity was maintained throughout.

2.2. ^{1}H NMR spectroscopy

Prior to NMR analysis, serum samples (150 μl) were diluted with 500 μl of 52% D_2O (Deuterium oxide, 99.9 at.% D, Aldrich Chemicals Company, South Africa) and 0.5% NaCl (Sigma Aldrich, South Africa) and placed in 5mm high quality NMR tubes (Sigma Aldrich., RSA). Conventional ^{1}H NMR spectra of each serum sample was measured at 300 MHz on a Varian Unity Inova using an IDG300-5EHT indirect detection gradient probe. The following pulse sequence was used: 90°–90°–RD–satdly–t_m–90°- acquire free induction decay (FID). RD represents a relaxation delay of 3.0 s, satdly was 1.5 s during which the water resonance was selectively irradiated, and the mixing time (t_m) was 0.4 ms. For each sample, 640 FIDS were collected with a spectral width of 4001.6 Hz and an acquisition time of 3.744 s. The acquired NMR spectra were collected for phase and baseline distortions and referenced to lipid $CH_2C{=}C$ at 1.89 ppm.

2.3. Data reduction of NMR data

The ^{1}H NMR spectra obtained for each serum sample (δ 4.0–0.4 ppm) were automatically data reduced to 120 integral segments of equal length (δ 0.03 ppm). Each segment consisted of the integral of the NMR region to which it was associated. Although the area in the suppression of the water resonance was not included, the region δ 6.0–4.4 was set to zero integral.

2.4. Statistical analysis

2.4.1. Standardization of the data

The original data was standardized by calculating a mean and standard deviation per individual, then subtracting the mean from each individual's amplitude and dividing the result by the standard deviation:

$$z_{fi} = \frac{a_{fi} - \bar{a}_i}{s_i}$$

where a_{fi} is the amplitude at frequency f for the ith individual; $\bar{a}_i$ the mean value of the 120 amplitudes for the ith individual; s_i the standard deviation of the 120 amplitudes for the ith individual; z_{fi} is the standardized amplitude at frequency f for the ith individual.

The result was that the standardized spectra for each individual had a mean of 0 and a standard deviation of 1.

2.4.2. Analysis of variance (ANOVA)

Comparison of the mean standardized spectra of the two groups (HIV/AIDS patients using ARV's and HIV negative individuals) was done by means of analysis of variance F-tests. This was done in order to establish whether or not there were significant differences between the groups. Only cases where the significance level (p-value) was less than 0.01 were considered.

2.4.3. Discriminant analysis

Linear discriminant analysis (LDA, [13]) was applied in order to classify a case as HIV positive using ARV's, HIV positive not using ARV's or HIV negative, given the NMR spectrum (standardized, as described above). Because there were amplitudes at 120 frequencies available, and using all of them would amount to gross overfitting, stepwise discriminant analysis was used to select the best set of frequencies for the classification. The resultant classification was based on 21 of the spectrum amplitudes. A cross-validation was done in which each case was omitted in turn from the analysis and then classified using the analysis based on the other 62 cases. The two discriminant functions, produced by this analysis, were plotted on a scatter diagram.

3. Results and discussion

Metabonomic techniques have been successfully employed in the identification and study of human disease [7–9]. As the

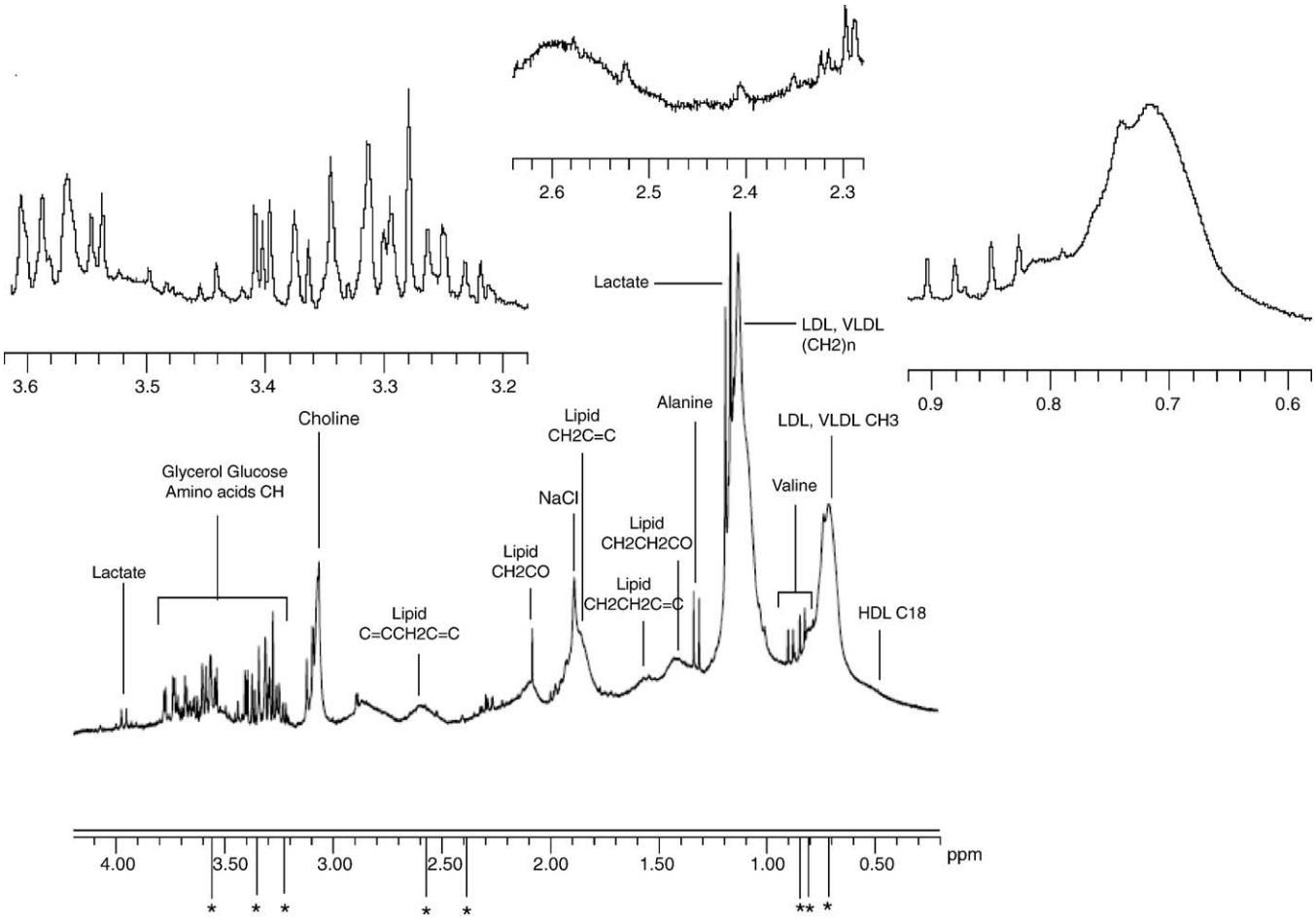

Fig. 1. A typical spectrum generated from 300 MHz ^{1}H NMR spectroscopy of serum from HIV negative individuals or HIV positive/AIDS patients using antiretroviral therapy. Eight segments identified through statistical analysis as exhibiting the most variation between the two sample groups are indicated with * and enlarged in the three expanded regions.

side effects of antiretroviral therapy are manifested through metabolic complications and disorders, metabonomic techniques may find extensive application in the monitoring of HIV positive/AIDS patients on antiretroviral treatment. To allow for this possibility it is necessary to determine whether the sera spectra of HIV positive/AIDS patients receiving treatment differs significantly from that of HIV-1 negative individuals. Results from this study suggest that distinction between the two sample groups is achievable and that the discernable segments of the spectra can draw parallels with known metabolic irregularities of treated HIV/AIDS patients.

To avoid bias based on the extent of disease progression, sera from HIV positive/AIDS patients with wide-ranging viral loads (from 1,000,000 to >25 copies/ml) were selected. ^{1}H NMR serum metabolite profiles of 29 HIV positive/AIDS patients utilizing anti-retrovirals and 29 HIV-1 negative were visually compared. The natural homeostatic control of serum was evident as spectra from the two populations exhibited similar peaks. Fig. 1 represents a typical 300 MHz NMR spectrum obtained from either of the two populations. Although NMR spectra produced from serum are complex, it has been established before that many resonances can be assigned directly based on their chemical shifts and signal multiplicities [14]. As such, the spectral peaks of Fig. 1 were assigned chemical compounds based on the findings of previously published literature [15–17]. Given the high information content and complexity of biological fluids, pattern recognition methods were needed for the interpretation of the NMR spectroscopy data. Results from the ANOVA analysis showed that the serum spectra of treated HIV positive/AIDS patients were discernable from those of healthy HIV-1 negative individuals. As stated previously, the NMR spectra for each serum sample were automatically data reduced to 120 integral segments of equal length. Of these 120 segments, exactly 30 were identified through ANOVA analysis as significantly dissimilar ($p < 0.01$). Many of these segments were adjacent to each other, for example each 0.03 ppm segment between 2.725 and 2.425 ppm was identified as significantly different between the two populations. Imposing a criterion of $p < 0.000$, eight segments were identified as exhibiting the most variation between the two spectra and are denoted in Fig. 1 by asterisks. The chemical content comprising some of these segments correlate with known metabolic irregularities of HIV/AIDS patients using antiretroviral drugs, i.e. the peaks within the segments contain lipids and glucose which are chemical compounds associated with ARV-associated disorders of lipodystrophy, hyperlipidaemia and hyperglycemia [10].

Table 1
Classification of samples as HIV-1 positive on antiretroviral therapy, HIV-1 positive not on antiretroviral therapy or HIV negative with original grouped cases and cross-validated grouped cases

	Group	Predicted group membership			Total
		HIV positive (ARV)	HIV negative	HIV positive (no ARV)	
Original[a]					
Count	HIV positive (ARV)	29	0	0	29
	HIV negative	1	28	0	29
	HIV positive (no ARV)	0	0	5	5
%	HIV positive (ARV)	100.0	0	0	100
	HIV negative	3.4	96.6	0	100
	HIV positive (no ARV)	0	0	100	100
Cross-validated[b]					
Count	HIV positive (ARV)	28	1	0	29
	HIV negative	2	27	0	29
	HIV positive (no ARV)	0	0	5	5
%	HIV positive (ARV)	96.6	3.4	0	100
	HIV negative	6.9	93.1	0	100
	HIV positive (no ARV)	0	0	100	100

[a] 98.4% of original grouped cases correctly classified.
[b] 95.2% of cross-validated grouped cases correctly classified. In cross-validation, each case is classified by the functions derived from all cases other than that case.

Linear discriminant analysis was then applied to the data in order to determine whether the samples could be classified as HIV positive or HIV negative. In this analysis, five samples from HIV/AIDS patients not using antiretroviral drugs were included. Both original and cross-validation analysis verified that classification of the samples into the three separate groups was possible. As shown in Table 1, correct group classification exceeded 95%. The resulting scatter diagram of the discriminant functions (Fig. 2) shows that the five HIV-1 cases without ARV treatment are separated from the other two groups on the vertical axis (the first discriminant function), while on the horizontal axis (the second discriminant function) the HIV-1 positive patients on ARV treatment and the HIV-1 negative patients are separated with the HIV-1 positive patients not on ARV treatment between these two groups. It is noticeable that one HIV negative case appears closer to the group of HIV positive individuals on ART. The individual in this case was not HIV positive at the time of testing, nor 3 months later when re-tested. The individual had no known metabolic or other medical condition and further investigation is underway to determine the underlying cause of this anomaly. The five HIV positive samples not on ARV treatment were included to illustrate that distinction can be made based on the use of ARV's. Although not the main focus of this study and using a small data set, these observations may also suggest the possibility of studying metabolic irregularities unrelated to anti-retroviral use.

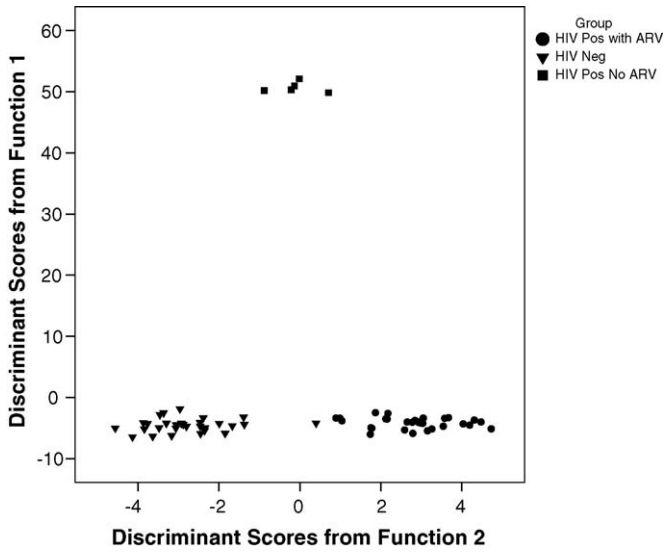

Fig. 2. Scatter plot of the discriminant functions for the three groups: 29 HIV-1 negative volunteers (▼), 29 HIV-1 positive patients taking anti-retroviral therapy (●) and five HIV-1 positive patients not on anti-retroviral therapy (■).

Most biomedical applications of metabonomics are performed at 600 MHz as opposed to the 300 MHz used in this study and it is possible that improved sensitivity may be achievable with the former. Nonetheless, this study shows that distinction based on metabolic profiles and chemometric analysis is possible and these preliminary findings suggest the use of NMR metabonomics in the monitoring of HIV/AIDS patients on antiretroviral therapy and may be expanded to the study of antiretroviral side-effects and the correlation of metabolic disorders.

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