Diagnosis of Type II Diabetes based on Non-glucose Regions of ^{1}H NMR Spectra of Urine

A metabonomic approach

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A NMR dataset with non-buffered urine samples consisting of 73 controls and 94 type II diabetes was subject to an in-house statistical classifier. A model was developed based on two glucose-free regions of the spectrum and those maximally discriminatory subregions selected most often by the algorithm were noted. The final classifier achieved 83.0% sensitivity and 83.6% specificity, with 83.2% overall accuracy. There were five spectral subregions selected by the algorithm as most relevant for discrimination. The protocol works well with non-buffered samples and has the potential for an automated clinical diagnosis of diabetes.

Keywords: NMR spectroscopy, urinary metabolites, diabetes, statistical classification, chemometry

Diabetes is a chronic life-threatening disease in which the body does not properly produce or respond to insulin, and is characterized by increased levels of blood glucose leading to severe damage to vital organs such as heart, eyes and kidneys. Traditional methods of monitoring the blood glucose concentration of an individual require that blood be taken by venipuncture. This method can be painful, inconvenient, and poses a risk of infection. A non-invasive method for measuring glucose involves urine analysis. However, glucose urine analysis may not reflect the correct status of the patient's blood glucose, because glucose appears in the urine only after a significant period with a presence of elevated levels of blood glucose. In diabetes, there may also be metabolic alterations in serum and urine other than the changes in the levels of glucose, and identification of such metabolites in urine could be useful in the development of non-invasive methods for the early diagnosis of diabetes [1].

Recently, the first two large scale epidemiological studies on long term effects (5 years) of intensive therapy for lowering glucose levels have shown contradictory results on the long term effects [2, 3]. Each of the studies was run on more than 10,000 participants, and both studies showed that near-normal glycemic control for three and a half to five years does not reduce cardiovascular events. Moreover, one of the studies revealed that the intensive glucose control via medication resulted in the increased risk of death from other causes, including cardiovascular [2]. These controversial findings will have to be addressed in the future with further evaluations of the reported data and also with further large scale trials. Regardless of how these epidemiological studies are assessed by the medical community in the future, the fact that glucose is not the only important parameter in the pathophysiology of diabetes is now widely accepted and the need for finding alternative markers to glucose, such as the method developed in this study, is crucial.

The possibility of quantifying glucose in urine by proton NMR spectroscopy was demonstrated as soon as high resolution NMR was applied to the study of intact body fluids [4-6]. Since a single NMR measurement can determine the concentration of other metabolites as well as glucose in a given sample, there is added value for the NMR approach in comparison with the usual tests [7-11]. Moreover, in order to diagnose diabetic patients with non-glucosuria, or in order to diagnose early stages of diabetes that will develop glucosuria at a later stage, it is highly important to develop diagnostic tests which rely on metabolites other than glucose [12, 13].

Over the years, in addition to glucose, other metabolites have been shown to be present at abnormal concentration levels in diabetic patients [14-17]. In spite of the potential for non-glucose NMR diagnosis of diabetes, previous reports in the literature revealed that although the averaged values for concentrations of various metabolites are different in diabetics and controls, the intervals over which the individual values are spread, overlap significantly [14-17]. This overlap of individual concentrations makes it impossible to develop a reliable clinical diagnosis of diabetes based on NMR-derived concentrations for metabolites other than glucose. Thus, averaged concentrations of metabolites may only be used for studies evaluating metabolic perturbation tendencies (e.g. for exploring the mechanism of the disease). It has become obvious that a reliable diagnostic test for diabetes based on NMR evaluation of metabolites other than glucose could be developed only with the help of a statistical classifier that would take into account either the whole spectrum, or at least large parts of the spectrum [18]. This has been

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successfully used in various other applications [18-21]. Considering (i) the huge number of people diagnosed worldwide with diabetes, thus the readily access to pathological samples, (ii) the current NMR techniques allowing fast, cheap and highly reproducible automated screenings, and (iii) the large number of algorithms for classifying spectra, the small number of published papers on ^{1}H NMR urine analysis of diabetic patients to date is quite surprising. Recent review covers the metabonomics aspects relevant for NMR diabetes studies [22].

Experimental part

Patient recruitment and sample collection

The study was approved by the Local Ethics Committee of the Diabetes, Nutrition and Metabolic Diseases Department of Craiova Clinical Emergency Hospital. The control group was made up of 73 subjects, 41 females and 32 males, characterized by a mean age of 35 years (ranging between 23 and 67 years). The type II DM group was made up of 107 patients, 58 females and 49 males, characterized by a mean age of 54 years (ranging between 25 and 75 years). The diagnostic criteria used for type 2 diabetes mellitus was according to the methods established by the American Diabetes Association [23]. These methods are (1) symptoms of diabetes plus a random plasma glucose level ≥ 200 mg/dL (11.1 mmol/L), (2) a fasting plasma glucose of ≥ 126 mg/dL (7.0 mmol/L) or (3) an oral tolerance test with a 2-h post-load (75 g glucose load) level ≥ 200 mg/dL (11.1 mmol/L). Any of these was confirmed on a subsequent day by one of the three methods. The patients had a history of type 2 diabetes mellitus for less than 5 years, were hospitalized in Craiova Emergency Clinical Hospital, receiving diabetic treatment and medical care. No special diet was imposed, apart from avoiding fish for 24 h before sample collection. The urine samples were collected between 10 a.m - 2 p.m in sterile containers with tight-fitting covers and further analyzed by an Abbott Analyzer for creatinine (alkaline picrate method) and urea nitrogen (urease method), and by semi-quantitative methods (Reagent Tests Strip for urobilinogen, *p*H, bilirubin, blood, ketone bodies, specific gravity, leukocyte, proteins, ascorbic acid, nitrite, and glucose). In the morning of the same day, patient blood samples were collected and analyzed for glucose, urea nitrogen and creatinine on an Abbott Analyzer. All these criteria were used to eliminate patients with possible urinary infection or renal involvement. The urine samples were frozen and stored at -79°C until ^{1}H-NMR analysis.

NMR Experiments

The NMR spectra were recorded on a Bruker Avance DRX 400 MHz spectrometer, using a 5 mm inverse detection multinuclear probe equipped with gradients on the z-axis. The samples were run in 5 mm Norell 507 NMR tubes. Before NMR analysis, the samples were allowed to reach room temperature (typically one hour) and centrifuged at 7,000 rpm for 10 min. To 0.9 mL urine, 0.1 mL of a stock solution of 5 mM sodium 3-(trimethylsilyl)-[2,2,3,3-d_4]-1-propionate (TSP) (Aldrich) in D_2O (Aldrich) was added. The pH was not adjusted. Most of the samples had pH values ranging from 5 to 6 with a few extremes between 6.5 and 7. The chemical shifts are reported as δ values (ppm) referenced to TSP as internal standard. The ^{1}H-NMR spectra were recorded with water presaturation. The pulse sequence used 32 scans, a 90° pulse, 30 s relaxation delay, 3 s CW irradiation, 4 s acquisition time, 4790 Hz spectral window, collecting 38 K data points, with a resolution of 0.13 Hz. Although the use of either no line broadening or 0.3 Hz line broadening is common in post-acquisition FID processing, we have chosen a value of 0.5 Hz. We found this value to be sufficient to resolve signals of interest and to perform proper assignment of resonances [16].

Data analysis

The spectra were converted from Bruker into ASCII format for both the real and imaginary parts. Magnitude spectra were derived from these and used in the analysis as this mode is less prone to phasing errors. For training the statistical classifier, only patients presenting glucosuria were used (94 out of 107 samples). The remaining 13 samples were set aside to test whether they would classify as DM once the classifier development was complete.

In order to exclude any interference with glucose (which if taken into account would give 100% accuracy) and eliminate possible operator errors induced with the addition of the TSP solution, the following two regions were used as inputs into the statistics program: 0.755 – 2.800 ppm (2,800 points) and 6.400 – 9.468 ppm (4,200 points). Within each of these regions, the spectra were normalized by dividing every data point by the total spectral area of the region. One may note that the excluded region is actually larger than the glucose chemical shifts region. This was done to exclude any possible artifacts due to the tailoring effect of the glucose signal in some of the high glucosoria patients, water suppression, and the broad and variable urea signal.

The statistical software was programmed in-house at the NRC Institute for Biodiagnostics (Winnipeg). A few maximally discriminatory subregions were selected from the input spectral regions using a genetic algorithm (GA)-driven optimization method [24]. Initially, a random set of subregions is selected. All data points are averaged within each subregion, and the resulting points are input to a linear discriminant analysis (LDA) classifier. As the GA process runs, mutation and crossover operations are performed on the region lists in an attempt to find subregions yielding higher LDA classification accuracy.

To improve classification reliability, the GA optimization process was run 50 times with each of 5 random splits of the data into training and monitoring sets. Each random split used 60 normal samples out of the 73 available and 75 diabetes samples out of the 94 available. The normals were given extra weight (1.25) in the calculations to help improve the balance in classification accuracy between the two classes. Each time, the GA was asked to find four discriminatory subregions. On completion of the GA process, histograms of the subregions selected were produced, and the subregions selected most-often were used in the final classifier, with LDA coefficients obtained using bootstrapping. For this final bootstrapping step, the dataset was split into training and test sets randomly 10,000 times and a weighted average of the sets of coefficients giving the highest classification performance on the test set was computed for use in the final classifier.

Results and discussions

A representative NMR spectrum of urine from a patient with DM2 is shown in figure 1. As can be seen from the shaded regions in the figure, there were five spectral subregions selected by the algorithm and used for the final classification: 8.09–8.01, 7.71–7.68, 2.67–2.60, 2.09–2.03, and 1.28–1.19 ppm. These regions were most commonly selected for each of the training/test splits. The respective histogram for each split showed portions of at least three and as many as five of these regions as most commonly

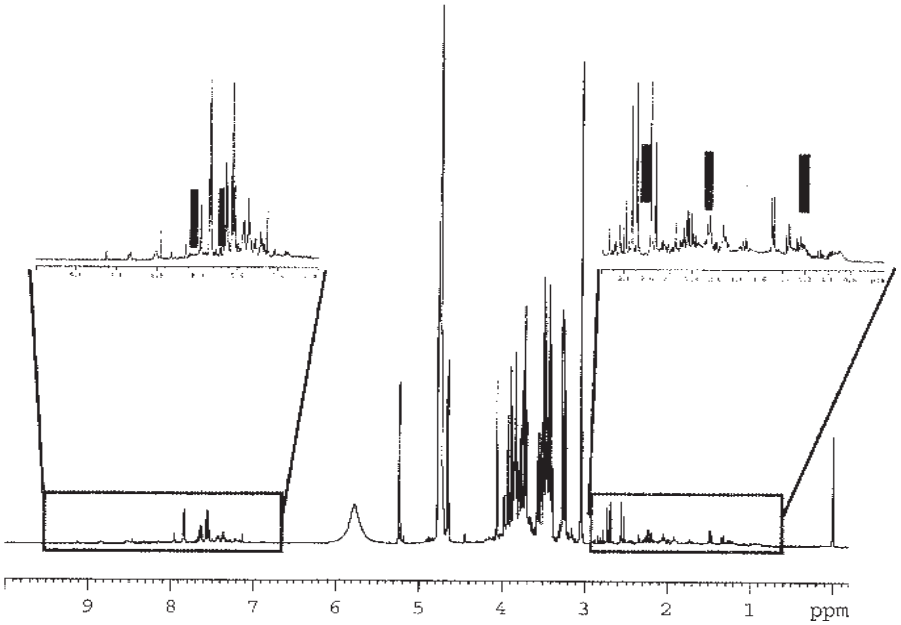

Fig. 1. ^{1}H-NMR spectrum (400 MHz, 25°C) of a urine sample belonging to the DM II group showing the two input subregions and the five discriminatory regions identified by the optimal region selection algorithm.

occurring. These regions could be assigned to several metabolites. However, our effort at this stage was primarily geared towards finding an accurate diagnostic test that works well in a blinded fashion without the need to know the identity of the discriminatory peaks. The final classifier after bootstrapping had 83.0% sensitivity and 83.6% specificity. There was also an additional set of 18 samples obtained from diabetic patients without glucosuria. When these spectra were subject to the classifier, 12 of the 18 were correctly classified as diabetes.

Detection of glucose in urine from diabetic patients cannot be a useful method for the early non-invasive diagnosis of diabetes mellitus, because we can only observe glucose in urine at a later stage when the serum glucose levels are very high. In the present study, we have used the non-glucose regions of the ^{1}H NMR spectra for the classification of urine samples from diabetic and non-diabetic subjects. These results are very encouraging, especially since they were obtained without resorting to the glucose signal. Moreover, the fact that 67% of the samples from the non-glucosuria subjects were correctly classified as DM suggests that our approach has the potential to detect early changes in the disease process.

Increases in relative concentrations of hydroxybutyrate which result from increased beta-oxidation of fatty acids have been observed previously in type 2 diabetic patients [14, 25]. Increases in hippurate concentration in type 2 diabetic patients have been observed previously [14, 26] but the exact mechanism of this has not been explained. Hippurate was not selected to be discriminatory in our study. Higher citrate concentrations have also been observed previously in DM patients [12, 14, 25] which was not the case in our study. This had been postulated to be due to increased citrate production in the tubular cells or/ and reduced citrate reabsorption from the tubular fluid because of glucose overflow. Moreover, acetate which has been found to be elevated in Type 2 DM in previous studies [12, 26] was not selected as discriminatory in the present study. The resonance due to trimethylamine-N-oxide (TMAO) was excluded from the analysis due to its proximity to the glucose signal. The main objective of the present study was merely to assess the diagnostic utility of such NMR-based analysis of urine from patients with diabetes. Hence, no extensive effort was made at this stage to evaluate individual metabolite concentrations and their role in the mechanism of the disease.

In previous studies, urine was subject to NMR analysis both with [12, 14, 27], and without [15, 17] *p*H adjustment. The pH adjustment has the advantage of simplifying the peak assignments as they would appear at the same chemical shift value. Analysis without *p*H adjustment avoids additional manipulations of the sample (limiting operator errors and reducing cost) and provides an extra parameter (chemical shift variation) for the chemometric treatments of the NMR data. Moreover, we found that adding a buffer solution to urine, produces in many cases a precipitate which has to be further removed from the sample. This behavior was also reported by other groups [12]. Thus, in the present study we have chosen not to adjust the sample *p*H.

At the current stage of the metabolomic/metabonomic development, with particular relevance to diabetes diagnosis, it is essential to push for more sample datasets, various magnetic field strengths, larger cohorts, alternative statistics classifiers and data processing approaches, in order to move away from the uncertainties related to small cohorts, stratification of particular populations, drug specific metabolites, overfitting of data, lack of cross-validation, and so on. In this respect it worth mentioning that recently two different groups published different statistical approaches and algorithms applied to the same datasets [12, 28, 29]. This approach of sharing row datasets should be highly encouraged for the sake of establishing the NMR based metabolomics as a validated diagnostic tool in medicine. In order to achieve this, we need more groups to publish their in-house data on both control and pathological groups. Our present study was aiming to contribute to filling this gap. Ideally, on long term, a unified sample preparation and NMR experimental protocol should be agreed by the metabolomic community. Meanwhile, differences in analytical protocols of various groups, particularly variations of samples *p*H should be coped with. Our statistical classifier was shown to work very well on non-buffered samples. Also, in comparison with previously described approaches [12, 13], our dataset was collected at lower magnetic field strength and the statistical classifier selected less discriminating regions.

Conclusion

We have developed a classifier for type II diabetes based on the non-glucose regions of the NMR spectrum that has

the potential for early diagnosis. The excellent predictive values of our model were obtained with minimal sample preparation. While it is possible that a more sophisticated sample preparation protocol may produce slightly improved discrimination results, we consider that keeping the sample preparation minimal allows the method to work better for individuals whose urine physico-chemical parameters deviate strongly from the average ones, and will be simpler to implement in the clinic. While it is likely that using buffered samples, would improve both the sensitivity and specificity of the method, the present algorithm proved very good results and should be also robust to data recorded in various laboratories and/or to samples with *p*H and ion concentrations that deviate strongly form the average ones. Furthermore, the basic preparation of samples, in addition to being faster and more convenient, is also less prone to operator errors. Our method may allow detection of the onset of type II diabetes before detectable levels of glucose are present in urine, leading to an earlier diagnosis of the onset of the disease. We intend to confirm this conclusion by studying a larger patient cohort.

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