Salivary molecular spectroscopy: a rapid and non-invasive monitoring tool for diabetes mellitus during insulin treatment

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Abstract:

Monitoring of blood glucose is an invasive, painful and costly practice in diabetes. Consequently, the search for a more cost-effective (reagent-free), non-invasive and specific diabetes monitoring method is of great interest. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy has been used in diagnosis of several diseases, however, applications in the monitoring of diabetic treatment are just beginning to emerge. Here, we used ATR-FTIR spectroscopy to evaluate saliva of non-diabetic (ND), diabetic (D) and diabetic 6U-treated of insulin (D6U) rats to identify potential salivary biomarkers related to glucose monitoring. The spectrum of saliva of ND, D and D6U rats displayed several unique vibrational modes and from these, two vibrational modes were pre-validated as potential diagnostic biomarkers by ROC curve analysis with significant correlation with glycemia. Compared to the ND and D6U rats, classification of D rats was achieved with a sensitivity of 100%, and an average specificity of 93.33% and 100% using bands 1452 cm^{-1} and 836 cm^{-1}, respectively. Moreover, 1452 cm^{-1} and 836 cm^{-1} spectral bands proved to be robust spectral biomarkers and highly correlated with glycemia (R^2 of 0.801 and 0.788, $P < 0.01$, respectively). Both PCA-LDA and HCA classifications achieved an accuracy of 95.2%. Spectral salivary biomarkers discovered using univariate and multivariate analysis may provide a novel robust alternative for diabetes monitoring using a non-invasive and green technology.

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia which results from insufficient secretion and/or reduced insulin action in peripheral tissues (Rolo e Palmeira, 2006; Ashcroft e Rorsman, 2012). According to the International Diabetes Federation (IDF), there are an estimated 425 million adults with diabetes worldwide, these include 212 million whom are estimated undiagnosed (IDF, 2017). Frequent monitoring of diabetes is essential for improved glucose control and to delay clinical complications related with diabetes. Besides, the early screening of DM is paramount to reduce the complications of this metabolic disorder worldwide (Uspstf, 2008). Despite being relatively invasive and painful, blood analysis per glucometer is currently feasible for screening, monitoring and diagnosing diabetes by needle finger punctures (Dowlaty *et al.*, 2013; Mascarenhas *et al.*, 2014). The constant need of piercing the fingers several times daily by most patients is inconvenient and may lead to the development of finger calluses and difficulty in obtaining blood samples (Dowlaty *et al.*, 2013).

Saliva reflects several physiological functions of the body (Desai e Mathews, 2014; Javaid *et al.*, 2016). In this way, salivary biomarkers might be an attractive alternative to blood for early detection, and for monitoring systemic diseases (Hu *et al.*, 2007). Among the advantages, saliva is simple to collect, non-invasive, convenient to store and, compared to blood, requires less handling during clinical procedures. Besides, saliva also contains analytes with real-time monitoring value which can be used to check the individuals condition (Javaid *et al.*, 2016; Zhang *et al.*, 2016). Currently, a broad set of methods are used to analyze saliva including immunoassays, colorimetric, enzymatic, kinetic, chromatographic and mass spectrometric analysis (Saxena *et al.*, 2017). Several studies showed higher salivary glucose levels in DM patients than non-hyperglycemic controls, which suggest that salivary glucose monitoring might be a useful in screening for diabetic patients. However, other studies reject the idea of a direct relationship between salivary glucose and glycemia (Mascarenhas *et al.*, 2014; Gupta, S. *et al.*, 2015; Nunes *et al.*, 2015; Naing e Mak, 2017). A main limitation of salivary-based measurement of glucose for diabetes monitoring is the presence of glucose in foods, which can disturb the monitoring process as it induces changes in salivary glucose concentration. Therefore, other alternatives of salivary monitoring should be studied.

Infrared (IR) spectroscopy is emerging as a powerful quantitative and qualitative technique for monitoring characterization of biological molecules in fluids (Bellisola e Sorio, 2012). Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy is a global, sensitive and highly reproducible physicochemical analytical technique that identifies structural molecules on the basis of their IR absorption(Ojeda e Dittrich, 2012). Considering that a biomolecule is determined by its unique structure, each one will exhibit a unique ATR-FTIR spectrum, representing the vibrational modes of the constituent structural bonds (Severcan *et al.*, 2010; Ojeda e Dittrich, 2012). ATR-FTIR is a green technology due to processes that eliminate the use of hazardous elements an overarching approach that is applicable to monitoring diseases. The IR spectral modes of biological samples, such as saliva, may be considered as biochemical fingerprints that correlate directly with the presence or absence of diseases, and, furthermore, provide the basis for the quantitative determination of several analytes for monitoring several diseases and to diagnostic interest (Khaustova *et al.*, 2010; Caetano Júnior *et al.*, 2015). The potential of salivary diagnostic for diabetes by IR spectroscopy using barium fluoride (BaF_2) slides was suggested previously (Scott *et al.*, 2010), however, the efficacy of DM monitoring in insulin-treated conditions using ultra-low volumes of saliva remains unknown.

In the present study, we tested the hypothesis that non-invasive spectral biomarkers can be identified in saliva of hyperglycemic diabetic and in insulin-treated diabetic rats, and the differentially expressed vibrational modes can be employed as salivary biomarkers for diabetes monitoring. Thus, the aim of our study was to identify infrared spectral signatures of saliva that are suitable to monitoring this metabolic disease in untreated and insulin-treated conditions. For this, the salivary vibrational modes profile of non-diabetic, diabetic and insulin-treated diabetic rats was quantitatively and qualitatively evaluated using univariate and multivariate analysis.

Results

Characterization of diabetes mellitus

To confirm the effectiveness of diabetes induction and insulin treatment, several parameters were assessed in anesthetized animals. As expected, to confirm the diabetic state, table 1 shows that diabetes reduced weight gain ($p < 0.05$), increased water intake ($p < 0.05$) and food ingestion ($p < 0.05$) compared with ND rats. Besides, in diabetic condition, higher plasma glucose ($p < 0.05$), as well as most pronounced urine volume ($p < 0.05$), associated with higher urine glucose concentration ($p < 0.05$), were observed in D rats compared with ND rats. Insulin treatment contributed to increased ($p < 0.05$)

weight gain and decreased water intake ($p < 0.05$) compared with placebo-treated D rats. As expected, insulin treatment decreased plasma glucose ($p < 0.05$), urine volume ($p < 0.05$) and urine glucose concentration compared with D rats. Glycemia and urine volume were similar ($p > 0.05$) in ND and D6U animals, indicating that insulin treatment completely reverted hyperglycemia and higher urine volume described in D rats. The insulin treatment promoted a strong reduction in the urinary glucose concentration; however, the urinary glucose concentration was increased ($p < 0.05$) in D6U compared to ND animals.

Average spectra of saliva

A representative infrared average spectrum of saliva from normoglycemic, hyperglycemic and insulin-treated conditions, which contains different molecules such as lipids, proteins, glycoproteins and nucleic acid, are represented in Figure 1. These salivary spectra indicated several differences among non-diabetic, diabetic and insulin-treated diabetic rats. Some bands of interest are shown in figure 1, which contains: asymmetric stretching vibration of CH_2 of acyl chains of lipids (2924 cm^{-1}); amide II (1549 cm^{-1}); asymmetric CH_3 bending modes of the methyl groups of proteins (1452 cm^{-1}); amide III band components of proteins (1313 cm^{-1}); mannose-6-phosphate and phosphorylated saccharide residue (1120 cm^{-1}) and C_2 conformation of sugar (836 cm^{-1}).

Spectral bands analyzed by IR spectroscopy

Spectral band areas that indicate the expression of specific molecules were analyzed in saliva. The band area values of 2924 cm^{-1}, 1549 cm^{-1}, 1313 cm^{-1}, 1120 cm^{-1} are presented in supplementary files. Herein, we showed two bands (1452 cm^{-1} and 836 $cm^{-}1$) with a higher potential for diabetes monitoring (Figure 2 and Figure 3, respectively). Representative spectra of 1452 cm^{-1} and 836 $cm^{-}1$ bands are depicted in Figure 2A and 3A. Diabetes induced a decrease ($p < 0.05$) at 1452 cm^{-1} and 836 cm^{-1} bands compared with non-diabetic rats, however, insulin-treated diabetic reverted this alteration in both bands (Figure 2B and 3B, respectively) .

To investigate whether these salivary vibrational modes would be reflective of glycemia regulation, these two salivary band areas were discovered to be, via univariate analysis, the best spectral candidates values to indicate the diabetes monitoring in samples with hyperglycemia, normoglycemia and under insulin treatment. Pearson's correlation between these spectral modes (1452 cm^{-1} and 836 cm^{-1}) with glycemia showed high correlation. The both salivary spectral bands presented strong negative correlation with r = -0.801; $p < 0.0001$ for 1452 cm^{-1} (Figure 2C) and r = -0.788; $p < 0.0001$ for 836 cm^{-1} (Figure 3C).

Considering that sensitivity and specificity are basic characteristics to determine the accuracy of diagnostic and monitoring test, ROC curve analysis were used to evaluate the potential diagnostic of these spectral bands under two conditions of analysis. The first one, we analyzed the condition of normoglycemic (ND and D6U) with hyperglycemic (D). The cutoff value to 1452 cm^{-1} band was 0.405, and the corresponding sensitivity and specificity were 100% and 93.3%, respectively. In ROC analysis, the area under the curve (AUC) of this band was 0.988 (Figure 2D). To emphasizes our focus on insulin-treated rats, we also showed ROC curve analysis comparing only D6U with D. Both sensitivity and specificity of 1452 cm^{-1} band was 100% with cutoff of 0.422 (p: 0.0027). Both sensitivity and specificity of 836 cm^{-1} band to differentiate normoglycemic (ND and D6U) than hyperglycemic (D) were 100% with cutoff of 0.128 (Figure 3D). As expected, the ROC curve to differentiate insulin-treated diabetic (D6U) than hyperglycemic (D) showed similar data (Figure 3E).

Differentiation among the groups by Principal Component Analysis followed by linear discriminant analysis (PCA-LDA) and Hierarchical Cluster Analysis (HCA)

Principal component analysis followed by linear discriminant analysis (PCA-LDA) was performed to reduce the dimensionality of the data set, with the preservation of the variance to evaluate the discrimination between the samples. PCA was performed using 6 principal components (PCs), accounting for 95.2% (20/21) of cumulative variance of correct classification with cross validation. The PCA model considered 95.8% of the data of the spectrum through the second derivative for analyze. After linear discriminant analysis (LDA) with leave-one-out cross-validation, three groups (ND, D and D6U) were formed, but only one sample belonging to class D6U was classified for group D (Figure 4). Supplementary table 1, Supplementary table 2 and Supplementary table 3 show the mean quadratic distance, discriminant linear function and the summary of classification of each sample (with quadratic distance of each sample, prediction, validation and probability), respectively, in saliva of ND, D and D6U rats.

Hierarchical cluster analysis (HCA) was performed to investigate the effects of treatment with insulin on diabetic to the differentiation of non-diabetic and diabetic samples. HCA was performed in part of salivary spectrum. The deconvolution analyzes were done in the five spectral regions represented in Figure 5, as A region (2995 cm^{-1} to 2889 cm^{-1}), B region (1664 cm^{-1} to 1581 cm^{-1}), C region (1410 cm^{-1} to 1234 cm^{-1}), D region (1149 cm^{-1} the 1080 cm^{-1}) and E region (1018 cm^{-1} to 955 cm^{-1}) which allowed the differentiation of the non-diabetic, diabetic and insulin-treated diabetic. As seen from the figure 5, all non-diabetics and diabetics were separate with 100% of discrimination. Only one insulin-treated diabetic was categorized as non-diabetic. The total accuracy, which is highly important for potential monitoring applications, was 95.2% (20/21).

Discussion

The development of a novel, rapid, noninvasive tool for the diagnosis, and the most important, for monitoring diabetes mellitus based on the comprehensive analysis of spectral salivary constituents would be of great use to health clinical. Herein, we have investigated the translational applicability of ATR-FTIR spectroscopy with potential monitoring of metabolic control in diabetes. Six potential spectral bands were detected by ATR-FTIR and, from these, two bands were showed a strong correlation with glycemia and high sensibility and specificity to differentiate hyperglycemic than normoglycemic conditions indicating potential monitoring applicability for diabetes. The discriminatory power of these two salivary ATR-FTIR bands area are candidates for monitoring diabetes under insulin therapy.

As expected in diabetic state, plasma glucose, urine volume and urine glucose concentration are increased in non-treated diabetic rats compared to non-diabetic rats. In addition, insulin treatment decreased glycemia, urine volume and urine glucose. These findings are consistent with other studies (Kusari *et al.*, 2007; Eleazu *et al.*, 2013),(Sabino-Silva *et al.*, 2009; Diniz Vilela *et al.*, 2016). It is known that salivary composition changes in diabetes mellitus (Rao *et al.*, 2009; Sabino-Silva *et al.*, 2013; Srinivasan *et al.*, 2015). Also, diabetes mellitus frequently decreases salivary flow, alters the expression of salivary proteins and increases glucose levels in saliva (Rao *et al.*, 2009; Bajaj *et al.*, 2012; Sabino-Silva *et al.*, 2013). From these parameters, it is possible to use salivary components to reflect the presence, and severity of hyperglycemia (Rao *et al.*, 2015). Saliva of diabetics with poor metabolic control shows an increase in salivary glucose concentration (Abd-Elraheem *et al.*, 2017). The correlation of glycemia with glucose concentration in saliva is still not well established, so currently it is not used to verify the degree of metabolic control and diagnosis in diabetes mellitus (Gupta, A. *et al.*, 2015; Kadashetti *et al.*, 2015; Puttaswamy *et al.*, 2017). ATR-FTIR spectroscopy has been used as an alternative discriminatory method to others chronic diseases, due to its major advantages of being label-free and non-destructive, rapid, high-throughput, not requiring sample preparation, and cost effective analytical method for providing details of the chemical composition and molecular structures (Simsek Ozek *et al.*, 2016; Yu *et al.*, 2017).

The spectral analysis method to dried saliva described in the present study may be used in rodent and human models. Spectral parameters, such as shifts in bands positions and changes in spectral modes intensity can be used to obtain valuable information about sample composition, which may have diagnostic and monitoring potential for many diseases (Severcan *et al.*, 2010). To get relative information about the concentration of the salivary molecules, integrated band area analysis was performed in the saliva spectra since, according to the Beer-Lambert law, absorption band intensity/band area is proportional to the concentration of the sample (Ozek et al. 2014; Turker et al. 2014). Therefore, differences in the band area for asymmetric CH_3 bending modes of the methyl groups of protein (1452 cm^{-1}) and C_2 endo/anti B-form helix conformation (836 cm^{-1}) differ in salivary constituents among the groups. Bencharit *et al.* (2013) showed the differences on composition of salivary proteins associated with metabolic control in diabetes on a proteomic analysis, and similar quantitative differences were found in the present study analyzed with spectroscopy ATR-FTIR. Type 2 diabetes mellitus induced changes in the lipid and protein components on the erythrocyte membrane and causing structural changes by FTIR spectroscopy in the protein secondary structure with change in the beta-sheet and beta-turn structures (Mahmoud, 2010).

These two salivary spectral modes showed a high and significant correlation with the metabolic control. Clinically, the most interesting comparisons are the correlation between these salivary spectral band areas and glycemia. Together, these salivary spectral bands showed a 100% of sensitivity and 100% of specificity in ROC analysis. ROC curve analysis is widely considered to be the most objective and statistically valid method for biomarker performance evaluation (Xia *et al.*, 2013). Regarding the potential for translation to the clinic, our results suggest that two salivary band areas, 1452 cm^{-1} and 836 cm^{-1} can be considered a non-invasive spectral biomarkers of monitoring diabetes treated with insulin. Different drug treatments and several levels of glucose concentration should ideally be possible to differentiate, therefore more studies need be investigated. These results indicate that these spectral modes can be used as a diagnostic and monitoring platform for diabetes mellitus, once interestingly, insulin treatment was also able to revert the salivary spectra observed in hyperglycemic state. Therefore, insulin treatment is not a potential confounding factor that may influence salivary vibrational mode in comparisons with glycemia. Some studies have indicated specific salivary biomarkers for diabetes, such as glucose, alpha-amylase, immunoglobulins, myeloperoxidases (Zloczower *et al.*, 2007; Rao *et al.*, 2009; Border *et al.*, 2012; Zhang *et al.*, 2016) with similar potential, but not with a focus on disease monitoring and/or with the use of IR spectroscopy.

Multivariate analysis as PCA-LDA and HCA can be used to discriminate samples based on their spectrum. In FTIR analysis the diagnostic accuracy for diabetes detection using saliva was 100.0% for the training set and 88.2% for the test (validation) set using linear discriminant analysis (LDA) calculations (Scott el al., 2010). However, in the present study both PCA-LDA and HCA obtained 95.2% of accuracy using saliva to discriminate normoglycemic, diabetic and insulin-treatment diabetic models. It is important emphasizes that our protocol used ultra-low values of saliva (2 µl) under airflow dried during only 2 minutes and the other study (Scott el al., 2010) used 50 µl (25 times greater) under dried during ~30 min at 25 Torr on 13 mm BaF windows. The analysis using univariate analysis was performed only in the present study. Besides, the Pearson's correlation between 1452 cm^{-1} and 836 cm^{-1} vibrational modes with glycemia described in present study showed higher correlation values ($r = 0.801$ and $r = -0.788$) comparing with another study (Scott el al., 2010; $r = 0.49$) using a SCN band, a classical indicator of tobacco smoking (a condition present in ~60% healthy and diabetic subjects).

Cluster analyses confirm its potential to discriminate ND, D and D6U groups with high accuracy. The success rate for ND e D was 100 %, and for D6U was 85.7%. Altogether, the data performed an accuracy of 95.23%. The inclusion of one sample of D6U animals in non-diabetic control group is expected considering that insulin is a gold-standard treatment of diabetes. We believe that this infrared analysis open perspectives to use saliva to monitor the metabolic control with molecules different than glucose. It is unequivocal that glucose is the main molecule to monitoring metabolic control in blood, however, the demonstration of glucose transporters in luminal membrane of ductal cells in salivary glands (Sabino-Silva *et al.*, 2013) highlight the need to evaluate other biomarkers in saliva.

Although we have shown that ATR-FTIR technology is useful for the identification of possible biomarkers for monitoring diabetes mellitus in the saliva of rats, this is a first exploratory study using ATR-FTIR technology for this purpose. Therefore, further studies are needed to validate the suggested spectral biomarkers in humans and to determine the applicability of this technique for the monitoring of diabetes mellitus in human saliva. It is important emphasizes that ATR-FTIR have been used for biofluids analysis, allowing same-day detection and grading of a range of diseases in humans (Hands *et al*.,2016; Hands *et al.*, 2014; Bonnier *et al.,* 2016; Khaustova *et al.,* 2010; Baker & Faulds, 2016; Smith *et al.*, 2016). Also, one limitation of this study is the inclusion of rats in higher levels of glycemia, which was not intentional but could be explained by effect of streptozotocin on beta cells.

In conclusion, we showed that ATR-FTIR spectroscopy in saliva is able to differentiate diabetic from non-diabetic and insulin-treated diabetic rats. Our data suggest specific fingerprint regions (highlighted two salivary spectral modes 1452 cm^{-1} and 836 cm^{-1}) capable of discriminating between hyperglycemic and normoglycemic conditions (insulin treated or not) in univariate analysis. A very high discriminatory accuracy of 95.2% was also obtained for classifying infrared spectra of saliva between diabetic, non-diabetic and insulin-treated rats by the PCA-LDA and HCA multivariate models. In summary, these salivary results indicate that ATR-FTIR spectroscopy coupled with univariate or multivariate chemometric analysis has the potential to provide a novel non-invasive approach to diabetes monitoring assisting medical decision making to avoid under-treatment or over-treatment with insulin.

Methods

Animals

This study was carried out in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian Society of Laboratory Animals Science (SBCAL). All experimental procedures for the handling, use and euthanasia were approved by the Ethics Committee for Animal Research of the Federal University of Uberlandia (UFU) (License #CEUA-UFU No. 013/2016) according to Ethical Principles adopted by the Brazilian College of Animal Experimentation (COBEA). All effort was taken to minimize the number of animals used and their discomfort.

Male wistar rats (~250g) were obtained from Center for Bioterism and Experimentation at the Federal University of Uberlandia. The animals were maintained under standard conditions (22 ± 1 °C, 60% ± 5% humidity and 12-hour light/dark cycles, light on at 7 AM) and were allowed with free access to standard diet and water at the Institute of Biomedical Sciences rodent housing facility.

Induction of Diabetes and insulin treatment

Animals were divided in Non-Diabetic (ND, n = 8), Diabetic (D, n = 6) and diabetic treated with 6U insulin (D6U, n = 7). Diabetes was induced in overnight-fasted animals by an intraperitoneal injection (60 mg/kg) of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO. USA) dissolved in 0.1 M citrate buffer (pH 4.5). Animals with hyperglycemia (>250 mg/dl) were chosen as diabetics. Non-diabetic animals received injection of NaCl 0.9% in similar volume.

Twenty one days later after induction of diabetes, diabetic rats were submitted to a 7-day treatment with vehicle (ND and D) or with 6U of insulin (D6U) per day (2U at 8:30 a.m. and 4U at 5:30 p.m.) subcutaneously (Sabino-Silva *et al.*, 2009). Glucose levels in overnight-fasted were obtained from the tail vein and measured using reactive strips (Accu-Chek Performa, Roche Diagnostic Systems, Basel, Switzerland) by a glucometer (Accu-Chek Performa, Roche Diagnostic Systems, Basel, Switzerland) in the moment of samples collection.

In the last day of treatment, the animals were kept in metabolic cages and water intake, food intake, urine volume were measured. Urine was collected over 24 h and the glucose concentration in the urine was evaluated using an enzymatic Kit (Labtest Diagnostica SA, Brazil). Besides that, variation of gain/loss body weight (Δ body weight) compared parameters in STZ or vehicle induction with parameters after insulin or vehicle treatment.

Saliva collection

After 7-days of treatment, the animals were anaesthetized by an intraperitoneal injection with ketamine (100 mg/kg) and xylazine (20 mg/kg). Stimulated saliva was collected with parasympathetic stimulation through pilocarpine injection (2 mg/kg, i.p.). Stimulated saliva was collected in pre weighed flasks for 10 min from the oral cavity (Sabino-Silva *et al.*, 2013). The collected saliva was stored at -80ºC for further processing and analysis.

Chemical profile in stimulated saliva by ATR-FTIR Spectroscopy

Salivary spectra were recorded in 3000 cm^{-1} to 400 cm^{-1} region using ATR-FTIR spectrophotometer Vertex 70 (Bruker Optics, Reinstetten, Germany) using a micro-attenuated total reflectance (ATR) component. The crystal material in ATR unit was a diamond disc as internal-reflection element. The salivary pellicle penetration depth ranges between 0.1 and 2 µm and depends on the wavelength, incidence angle of the beam and the refractive index of ATR-crystal material. In the ATR-crystal the infrared beam is reflected at the interface toward the sample. Saliva was directly dried using airflow on ATR-crystal for 2 min before salivary spectra recorded. The air spectra was used as a background in ATR-FTIR analysis. Sample spectra and background was taken with 4 cm^{-1} of resolution and 32 scans were performed for salivary analysis.

Spectra data evaluation procedures

The spectra data obtained were processed using Opus 6.5 software (Bruker Optics, Reinstetten, Germany). Measurements were performed in mid-infrared region (3000–400 cm^{-1}) with spectral resolution of 4 cm^{-1} and 32 scans per spectrum. Samples were pressed into ATR diamond crystal with standardized pressure. For the generation of mean spectra and band areas, the spectra were normalized by vector and baseline corrected to avoid errors during the sample preparations and spectra analysis. To evaluate the mean values for the peak positions, band area of the spectra were considered belonging to each animal of the groups. The band positions were measured using the frequency corresponding to the center of weight of each band. Band areas were calculated from normalized and baseline corrected spectra using OPUS software. Sensitivity and specificity values were calculated based on the external test set as follows:

The specificity or true negative rate is defined as the percentage of rats who are correctly identified as being normoglycemic Non-Diabetic (ND) or normoglycemic diabetic treated with 6U insulin (D6U:

$$\text{Specificity} = \frac{TN}{TN + FP}$$

The quantity 1-specificity is the false positive rate and is the percentage of rats that are incorrectly identified as diabetic (D).

The sensitivity or true positive rate is defined as the percentage of rats who are correctly identified as diabetic (D):

$$\text{Sensitivity} = \frac{TP}{TP + FN}$$

where TP stands for true positives; TN for true negatives; FP for false positives; and FN for false negatives.

Principal component analysis followed by linear discriminant analysis (PCA-LDA) and Hierarchical Cluster Analysis (HCA)

The principal components were calculated using a full range of the FT-IR spectra (ND, D and DU6) between 3700 and 500 cm-1, and a covariance matrix. The first step was normalization followed by mean centering, the data were analyzed using the principal components analysis (PCA). In this study, the first six principal components (PC1-PC6) were used to perform the linear discriminant analysis (LDA) with leave-one-out cross-validation, according to the pathological reports.

Infrared spectra of saliva samples were also analyzed by OPUS software (version 4.2) using hierarchical cluster analysis with first-derivative of the training data set. The Dendrogram was performed by Ward's clustering algorithm in the defined spectral regions.

Statistical analysis

The data of the band area were analyzed using the one-way analysis of variance (ANOVA), followed by Tukey Multiple Comparison as a *post-hoc* test. The correlation between values of blood glucose concentration and salivary band areas of the spectra were analyzed by the Pearson correlation test. For all spectral band candidates, we constructed the Receiver Operating Characteristic (ROC) curve and computed the area under the curve (AUC) value, sensitivity and specificity by numerical integration of the ROC curve. The Kolmogorov-Smirnov test was applied to test the normality of the variables. All these analyses were performed using the software GraphPad Prism (GraphPad Prism version 7.00 for Windows, GraphPad Software, San Diego, CA, USA). Only values of $p < 0.05$ were considered significant and the results were expressed as mean ± S.D.

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References

ABD-ELRAHEEM, S. E.; EL SAEED, A. M.; MANSOUR, H. H. Salivary changes in type 2 diabetic patients. **Diabetes Metab Syndr,** v. 11 Suppl 2, p. S637-s641, Dec 2017. ISSN 1871-4021.

ASHCROFT, F. M.; RORSMAN, P. Diabetes mellitus and the beta cell: the last ten years. **Cell,** v. 148, n. 6, p. 1160-71, Mar 16 2012. ISSN 0092-8674.

BAJAJ, S. et al. Oral manifestations in type-2 diabetes and related complications. **Indian J Endocrinol Metab,** v. 16, n. 5, p. 777-9, Sep 2012. ISSN 2230-9500.

BAKER, Matthew J.; FAULDS, Karen. Fundamental developments in clinical infrared and Raman spectroscopy. **Chemical Society Reviews**, v. 45, n. 7, p. 1792-1793, Apr 2016. ISSN 0306-0012

BELLISOLA, G.; SORIO, C. Infrared spectroscopy and microscopy in cancer research and diagnosis. **Am J Cancer Res,** v. 2, n. 1, p. 1-21, 2012. ISSN 2156-6976.

BENCHARIT, S. et al. Salivary proteins associated with hyperglycemia in diabetes: a proteomic analysis. **Molecular bioSystems,** v. 9, n. 11, p. 2785-2797, 2013. ISSN 1742-2051

BONNIER, Franck et al. Screening the low molecular weight fraction of human serum using ATR-IR spectroscopy. **Journal of biophotonics**, v. 9, n. 10, p. 1085-1097, Oct 2016. ISSN: 1864-063X

BORDER, M. B. et al. Exploring salivary proteomes in edentulous patients with type 2 diabetes. **Mol Biosyst,** v. 8, n. 4, p. 1304-10, Apr 2012. ISSN 1742-2051.

CAETANO JÚNIOR, P. C.; STRIXINO, J. F.; RANIERO, L. Analysis of saliva by Fourier transform infrared spectroscopy for diagnosis of physiological stress in athletes. **Research on Biomedical Engineering,** v. 31, p. 116-124, 2015. ISSN 2446-4740. Disponível em: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S2446-47402015000200116&nrm=iso >.

DESAI, G. S.; MATHEWS, S. T. Saliva as a non-invasive diagnostic tool for inflammation and insulin-resistance. **World J Diabetes,** v. 5, n. 6, p. 730-8, Dec 15 2014. ISSN 1948-9358 (Print) 1948-9358.

DINIZ VILELA, D. et al. The Role of Metformin in Controlling Oxidative Stress in Muscle of Diabetic Rats. **Oxid Med Cell Longev,** v. 2016, p. 6978625, 2016. ISSN 1942-0994. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/27579154 >.

DOWLATY, N.; YOON, A.; GALASSETTI, P. Monitoring states of altered carbohydrate metabolism via breath analysis: are times ripe for transition from potential to reality? **Curr Opin Clin Nutr Metab Care,** v. 16, n. 4, p. 466-72, Jul 2013. ISSN 1363-1950.

ELEAZU, C. O. et al. Ameliorative Potentials of Ginger (Z. officinale Roscoe) on Relative Organ Weights in Streptozotocin induced Diabetic Rats. **Int J Biomed Sci,** v. 9, n. 2, p. 82-90, Jun 2013. ISSN 1550-9702 (Print) 1550-9702.

GUPTA, A. et al. Evaluation of Correlation of Blood Glucose and Salivary Glucose Level in Known Diabetic Patients. **J Clin Diagn Res,** v. 9, n. 5, p. Zc106-9, May 2015. ISSN 2249-782X (Print)

0973-709x.

GUPTA, S. et al. Comparison of salivary and serum glucose levels in diabetic patients. **J Diabetes Sci Technol,** v. 9, n. 1, p. 91-6, Jan 2015. ISSN 1932-2968.

HANDS, James R. et al. Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectral discrimination of brain tumour severity from serum samples. **Journal of biophotonics**, v. 7, n. 3-4, p. 189-199, Apr 2014.

HANDS, James R. et al. Brain tumour differentiation: rapid stratified serum diagnostics via attenuated total reflection Fourier-transform infrared spectroscopy. **Journal of neuro-oncology**, v. 127, n. 3, p. 463-472, May 2016. ISSN 1573-7373 (Print) 0167-594X

HU, S.; LOO, J. A.; WONG, D. T. Human saliva proteome analysis and disease biomarker discovery. **Expert Rev Proteomics,** v. 4, n. 4, p. 531-8, Aug 2007. ISSN 1478-9450.

JAVAID, M. A. et al. Saliva as a diagnostic tool for oral and systemic diseases. **J Oral Biol Craniofac Res,** v. 6, n. 1, p. 66-75, Jan-Apr 2016. ISSN 2212-4268 (Print) 2212-4268.

KADASHETTI, V. et al. Glucose Level Estimation in Diabetes Mellitus By Saliva: A Bloodless Revolution. **Rom J Intern Med,** v. 53, n. 3, p. 248-52, Jul-Sep 2015. ISSN 1220-4749 (Print)

1220-4749.

KHAUSTOVA, S. et al. Noninvasive biochemical monitoring of physiological stress by Fourier transform infrared saliva spectroscopy. **Analyst,** v. 135, n. 12, p. 3183-92, Dec 2010. ISSN 0003-2654.

KUSARI, J. et al. Effect of memantine on neuroretinal function and retinal vascular changes of streptozotocin-induced diabetic rats. **Invest Ophthalmol Vis Sci,** v. 48, n. 11, p. 5152-9, Nov 2007. ISSN 0146-0404 (Print) 0146-0404.

MAHMOUD, S. S. The impact of elevated blood glycemic level of patients with type 2 diabetes mellitus on the erythrocyte membrane: FTIR study. **Cell Biochem Biophys,** v. 58, n. 1, p. 45-51, Sep 2010. ISSN 1085-9195.

MASCARENHAS, P.; FATELA, B.; BARAHONA, I. Effect of diabetes mellitus type 2 on salivary glucose--a systematic review and meta-analysis of observational studies. **PLoS One,** v. 9, n. 7, p. e101706, 2014. ISSN 1932-6203.

NAING, C.; MAK, J. W. Salivary glucose in monitoring glycaemia in patients with type 1 diabetes mellitus: a systematic review. **J Diabetes Metab Disord,** v. 16, p. 2, 2017. ISSN 2251-6581 (Print) 2251-6581.

NUNES, L. A.; MUSSAVIRA, S.; BINDHU, O. S. Clinical and diagnostic utility of saliva as a non-invasive diagnostic fluid: a systematic review. **Biochem Med (Zagreb),** v. 25, n. 2, p. 177-92, 2015. ISSN 1330-0962 (Print)

1330-0962.

OJEDA, J. J.; DITTRICH, M. Fourier transform infrared spectroscopy for molecular analysis of microbial cells. **Methods Mol Biol,** v. 881, p. 187-211, 2012. ISSN 1064-3745.

PUTTASWAMY, K. A.; PUTTABUDHI, J. H.; RAJU, S. Correlation between Salivary Glucose and Blood Glucose and the Implications of Salivary Factors on the Oral Health Status in Type 2 Diabetes Mellitus Patients. **J Int Soc Prev Community Dent,** v. 7, n. 1, p. 28-33, Jan-Feb 2017. ISSN 2231-0762 (Print)

2231-0762.

RAO, P. V. et al. Salivary protein glycosylation as a noninvasive biomarker for assessment of glycemia. **J Diabetes Sci Technol,** v. 9, n. 1, p. 97-104, Jan 2015. ISSN 1932-2968.

RAO, P. V. et al. Proteomic identification of salivary biomarkers of type-2 diabetes. **J Proteome Res,** v. 8, n. 1, p. 239-45, Jan 2009. ISSN 1535-3893 (Print) 1535-3893.

ROLO, A. P.; PALMEIRA, C. M. Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. **Toxicol Appl Pharmacol,** v. 212, n. 2, p. 167-78, Apr 15 2006. ISSN 0041-008X (Print) 0041-008x.

SABINO-SILVA, R. et al. Na+-glucose cotransporter SGLT1 protein in salivary glands: potential involvement in the diabetes-induced decrease in salivary flow. **J Membr Biol,** v. 228, n. 2, p. 63-9, Mar 2009. ISSN 0022-2631.

SABINO-SILVA, R. et al. Increased SGLT1 expression in salivary gland ductal cells correlates with hyposalivation in diabetic and hypertensive rats. **Diabetol Metab Syndr,** v. 5, n. 1, p. 64, Oct 24 2013. ISSN 1758-5996 (Print) 1758-5996.

SAXENA, S. et al. A Review of Salivary Biomarker: A Tool for Early Oral Cancer Diagnosis. **Adv Biomed Res,** v. 6, p. 90, 2017. ISSN 2277-9175 (Print)

2277-9175.

SCOTT, D. A. et al. Diabetes-related molecular signatures in infrared spectra of human saliva. **Diabetol Metab Syndr,** v. 2, p. 48, Jul 14 2010. ISSN 1758-5996.

SEVERCAN, F. et al. FT-IR spectroscopy in diagnosis of diabetes in rat animal model. **J Biophotonics,** v. 3, n. 8-9, p. 621-31, Aug 2010. ISSN 1864-063x.

SIMSEK OZEK, N. et al. Differentiation of Chronic and Aggressive Periodontitis by FTIR Spectroscopy. **J Dent Res,** v. 95, n. 13, p. 1472-1478, Dec 2016. ISSN 0022-0345.

SMITH, Benjamin R. et al. Combining random forest and 2D correlation analysis to identify serum spectral signatures for neuro-oncology. **Analyst**, v. 141, n. 12, p. 3668-3678, Jun 2016.

SRINIVASAN, M. et al. Literature-based discovery of salivary biomarkers for type 2 diabetes mellitus. **Biomark Insights,** v. 10, p. 39-45, 2015. ISSN 1177-2719 (Print) 1177-2719.

USPSTF. Screening for type 2 diabetes mellitus in adults: U.S. Preventive Services Task Force recommendation statement. **Ann Intern Med,** v. 148, n. 11, p. 846-54, Jun 3 2008. ISSN 0003-4819.

XIA, J. et al. Translational biomarker discovery in clinical metabolomics: an introductory tutorial. **Metabolomics,** v. 9, n. 2, p. 280-299, Apr 2013. ISSN 1573-3882 (Print)

1573-3882.

YU, M. C. et al. Label Free Detection of Sensitive Mid-Infrared Biomarkers of Glomerulonephritis in Urine Using Fourier Transform Infrared Spectroscopy. **Sci Rep,** v. 7, n. 1, p. 4601, Jul 04 2017. ISSN 2045-2322.

ZHANG, C. Z. et al. Saliva in the diagnosis of diseases. **Int J Oral Sci,** v. 8, n. 3, p. 133-7, Sep 29 2016. ISSN 1674-2818.

ZLOCZOWER, M. et al. Relationship of flow rate, uric acid, peroxidase, and superoxide dismutase activity levels with complications in diabetic patients: can saliva be used to diagnose diabetes? **Antioxid Redox Signal,** v. 9, n. 6, p. 765-73, Jun 2007. ISSN 1523-0864 (Print) 1523-0864.

Table 1. Effect of diabetes and insulin on body weight, water intake, food intake, glycemia, urine volume and urine glucose concentration.

Parameters	ND	D	D6U
Δ Body weight (g)	48.4±8.3	-2.7±11.3*	39.5±12.8#
Water intake (mL)	39.1±3.1	150.6±17.9*	60.0±6.8#
Food intake (g)	18.3±1.3	35.0±4.1*	29.7±2.6*
Glycemia (mg/dL)	83.2±4.2	497.6±19.6*	81.0±19.2#
Urine volume (mL)	22.1.6±3.4	128.9±8.6*	40.7±7.1#
Urine glucose (mg/dL)	24.7±7.2	337.2±15.8*	148.0±34.6*#

Supplementary Table 1. Mean quadratic distance in saliva of ND, D and D6U rats.

Quadratic distance	ND	D	D6U
D	0,0000	23,3348	37,2085
D6U	23,3348	0,0000	11,5541
ND	37,2085	11,5541	0,0000

Supplementary Table 2. Discriminant linear function in saliva of ND, D and D6U rats.

	ND	D	D6U
Constant	-7,105	-1,663	-3,374
CP1	20,686	1,288	-16,659
CP2	34,740	-8,064	-19,007
CP3	18,897	-0,100	-14,095
CP4	-3,054	5,305	-2,359
CP5	4,356	-9,836	5,357
CP6	5,835	-0,779	-3,699

Supplementary Table 3. Summary of classification with the quadratic distance of each sample, prediction, validation and probability of each sample in saliva of ND, D and D6U rats.

					Quadratic distance		Probability	
Sample	True group	Predicted. group	Val-X Group	Group	Pred.	Val-X	Predicted	Val-X
1	ND	ND	ND	D	58.073	72.570	0.00	0.00
				D6U	23.160	29.507	0.00	0.00
				ND	6.079	12.211	1.00	1.00
2	ND	ND	ND	D	36.580	34.838	0.00	0.00
				D6U	11.348	11.078	0.03	0.14
				ND	4.371	7.464	0.97	0.86
3	ND	ND	ND	D	35.359	33.417	0.00	0.00
				D6U	10.335	9.816	0.02	0.07
				ND	2.961	4.497	0.98	0.93
4	ND	ND	ND	D	33.837	31.958	0.00	0.00
				D6U	20.628	22.550	0.00	0.00
				ND	3.528	5.608	1.00	1.00
5	ND	ND	ND	D	63.675	88.572	0.00	0.00
				D6U	34.276	54.739	0.00	0.00
				ND	6.646	14.182	1.00	1.00
6	ND	ND	ND	D	29.741	28.369	0.00	0.00
				D6U	9.677	9.203	0.05	0.16
				ND	3.678	5.918	0.95	0.84
7	ND	ND	ND	D	40.529	38.586	0.00	0.00
				D6U	8.646	8.220	0.02	0.03
				ND	1.065	1.409	0.98	0.97
8	ND	ND	ND	D	31.222	29.651	0.00	0.00
				D6U	5.711	5.542	0.21	0.39
				ND	3.021	4.612	0.79	0.61
9	D	D	D	D	6.582	15.951	1.00	1.00
				D6U	26.329	27.438	0.00	0.00
				ND	42.640	44.320	0.00	0.00
10	D	D	D	D	6.150	14.176	1.00	1.00
				D6U	35.688	42.839	0.00	0.00
				ND	41.710	42.420	0.00	0.00
11	D	D	D	D	4.543	8.862	1.00	1.00
				D6U	24.542	23.925	0.00	0.00
				ND	41.537	41.006	0.00	0.00
12	D	D	D	D	5.014	10.244	1.00	0.99
				D6U	21.611	20.666	0.00	0.01

				ND	41.594	41.372	0.00	0.00
13	D	D	D	D	5.526	11.899	1.00	0.92
				D6U	17.901	16.907	0.00	0.08
				ND	28.636	27.276	0.00	0.00
14	D	D	D	D	9.967	40.402	1.00	1.00
				D6U	51.721	117.859	0.00	0.00
				ND	64.915	127.897	0.00	0.00
15	D6U	D6U	D6U	D	45.891	67.075	0.00	0.00
				D6U	6.835	15.774	1.00	1.00
				ND	22.101	29.173	0.00	0.00
16	D6U	D6U	D6U	D	34.031	36.870	0.00	0.00
				D6U	4.261	7.567	0.88	0.54
				ND	8.291	7.852	0.12	0.46
17	D6U	D6U	D6U	D	31.428	32.102	0.00	0.00
				D6U	3.134	5.055	0.93	0.79
				ND	8.210	7.754	0.07	0.21
18	D6U	D6U	D6U	D	23.558	22.563	0.00	0.00
				D6U	3.718	6.296	0.97	0.87
				ND	10.479	10.037	0.03	0.13
19	D6U	D6U	D6U	D	23.534	22.983	0.00	0.00
				D6U	5.454	10.844	1.00	1.00
				ND	24.905	31.891	0.00	0.00
20	D6U	D6U	D6U	D	29.999	30.895	0.00	0.00
				D6U	4.318	7.707	1.00	1.00
				ND	19.325	21.357	0.00	0.00
21	D6U	D6U	D*	D	13.77	13.15	0.21	1.00
				D6U	11.15	51.72	0.79	0.00
				ND	26.44	62.40	0.00	0.00

Legends

Figure 1. Average ATR-FTIR spectra (3000-400 cm-1) in saliva of Non-Diabetic rats (ND), diabetic rats (D) and diabetic treated with 6U insulin (D6U).

Figure 2. Spectral of 1452 cm-1 (A); Band area of 1452 cm-1 (B); Pearson correlation between glycemia and band area of 1452 cm-1 (C); ROC curve analyses of 1452 to

normoglycemic and hyperglycemic (D); ROC curve analyses of 1452 to diabetic and diabetic treated with insulin (E). Non-diabetic rats (ND), diabetic rats (D) and diabetic treated with 6U insulin (D6U).

Figure 3. Spectral of 836 cm-1 (A); Band area of 836 cm-1 (B); Pearson correlation between glycemia and band area of 836 cm-1 (C); ROC curve analyses of 836 to normoglycemic and hyperglycemic (D); ROC curve analyses of 836 to diabetic and diabetic treated with insulin (E). Non-diabetic rats (ND), diabetic rats (D) and diabetic treated with 6U insulin (D6U).

Figure 4. PCA analyses. Non-diabetic rats (ND), diabetic rats (D) and diabetic treated with 6U insulin (D6U).

Figure 5. HCA analyses. Non-diabetic rats (ND), diabetic rats (D) and diabetic treated with 6U insulin (D6U).

Supplementary Figure 1. Spectral of 2924 cm-1 (A); Band area of 2924 cm-1 (B); Pearson correlation between glycemia and band area of 2924 cm-1 (C); ROC curve analyses of 2924 cm-1 to normoglycemic and hyperglycemic (D); ROC curve analyses of 2924 cm-1 to diabetic and diabetic treated with insulin (E). Non-diabetic rats (ND), diabetic rats (D) and diabetic treated with 6U insulin (D6U).

Supplementary Figure 2. Spectral of 1549 cm-1 (A); Band area of 1549 cm-1 (B); Pearson correlation between glycemia and band area of 1549 cm-1 (C); ROC curve analyses of 1549 cm-1 to normoglycemic and hyperglycemic (D); ROC curve analyses of 1549cm-1 to diabetic and diabetic treated with insulin (E). Non-diabetic rats (ND), diabetic rats (D) and diabetic treated with 6U insulin (D6U).

Supplementary Figure 3. Spectral of 1313 cm-1 (A); Band area of 1313 cm-1 (B); Pearson correlation between glycemia and band area of 1313 cm-1 (C); ROC curve analyses of 1313 cm-1 to normoglycemic and hyperglycemic (D); ROC curve analyses of 1313 cm-1 to diabetic and diabetic treated with insulin (E). Non-diabetic rats (ND), diabetic rats (D) and diabetic treated with 6U insulin (D6U).

Supplementary Figure 4. Spectral of 1120 cm-1 (A); Band area of 1120 cm-1 (B); Pearson correlation between glycemia and band area of 1120 cm-1 (C); ROC curve analyses of 1120 cm-1 to normoglycemic and hyperglycemic (D); ROC curve analyses of 1120 cm-1 to diabetic and diabetic treated with insulin (E). Non-diabetic rats (ND), diabetic rats (D) and diabetic treated with 6U insulin (D6U).

Figure 1.

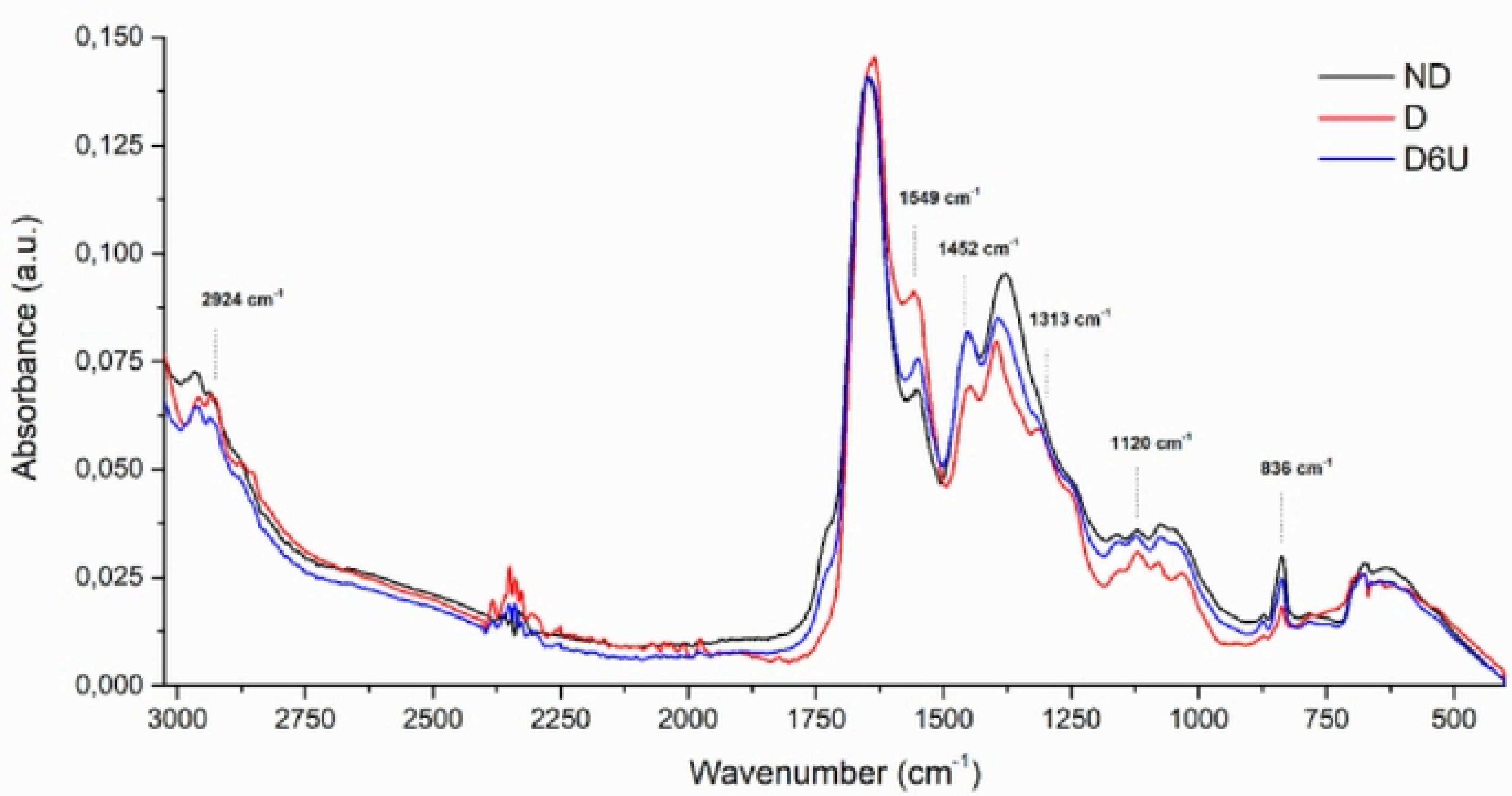
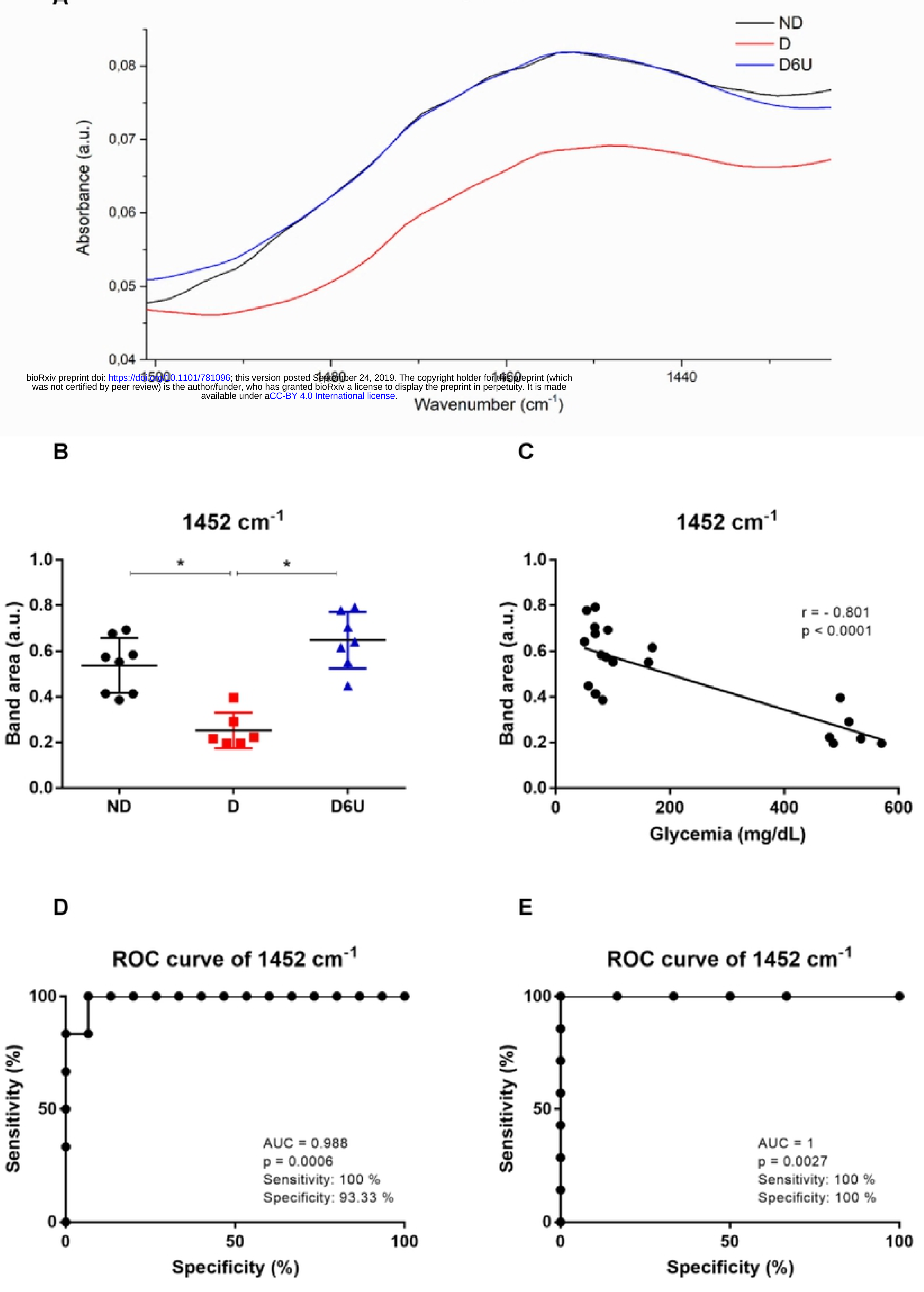

ND
D
D6U
2924 cm-1
1549 cm-1
1452 cm-1
1313 cm-1
1120 cm-1
836 cm-1
Absorbance (a.u.)
0,000
0,025
0,050
0,075
0,100
0,125
0,150
3000
2750
2500
2250
2000
1750
1500
1250
1000
750
500
Wavenumber (cm-1)

Figure 2.



A
ND
D
D6U
Absorbance (a.u.)
0,08
0,07
0,06
0,05
0,04
1500
1480
1460
1440
Wavenumber (cm-1)
B
1452 cm-1
Band area (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
ND
D
D6U
C
1452 cm-1
r = - 0.801
p < 0.0001
Band area (a.u.)
0
200
400
600
Glycemia (mg/dL)
D
ROC curve of 1452 cm-1
Sensitivity (%)
100
50
0
AUC = 0.988
p = 0.0006
Sensitivity: 100 %
Specificity: 93.33 %
Specificity (%)
E
ROC curve of 1452 cm-1
AUC = 1
p = 0.0027
Sensitivity: 100 %
Specificity: 100 %
Specificity (%)

Figure 3.

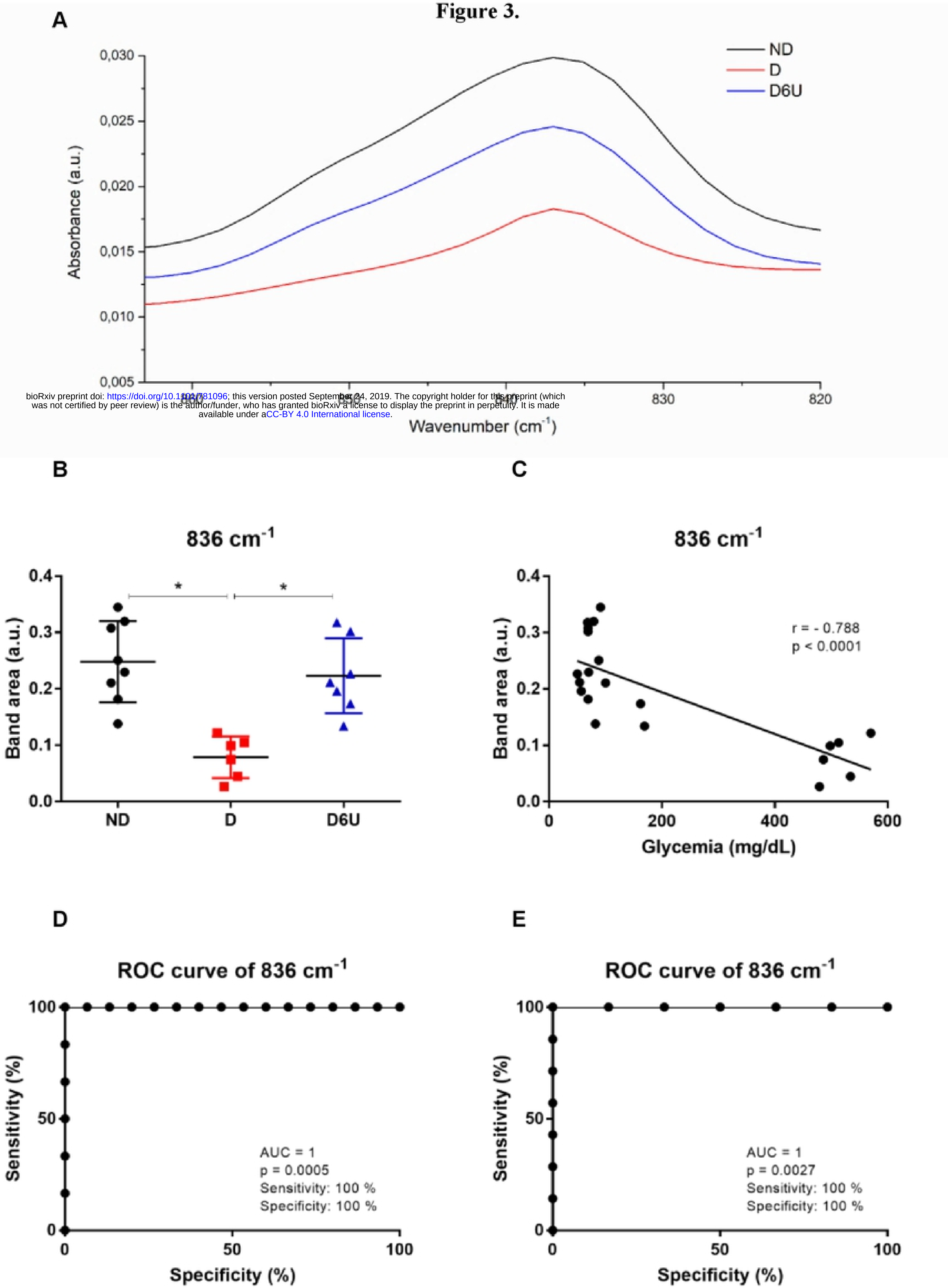
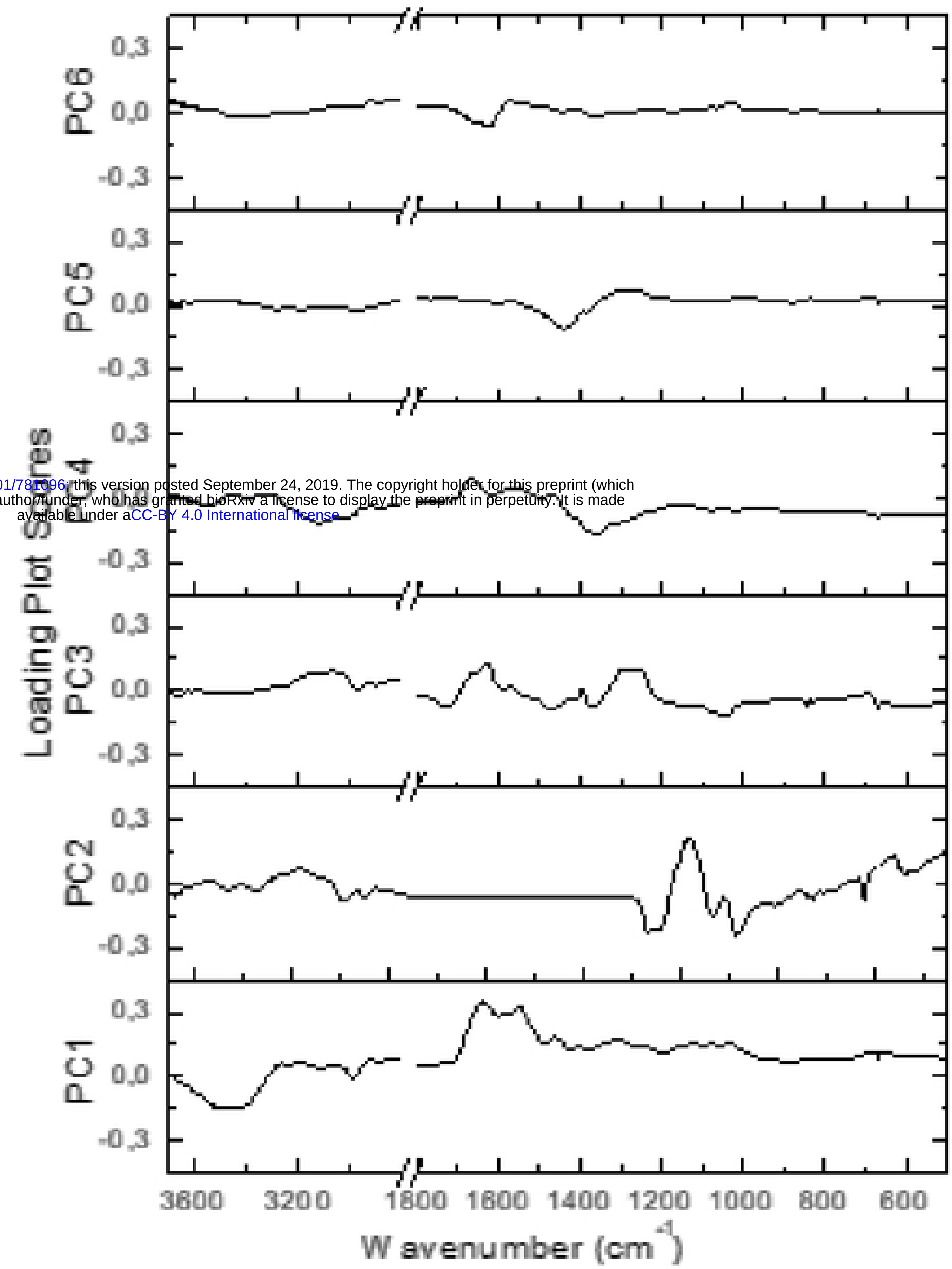

A
ND
D
D6U
Absorbance (a.u.)
0,030
0,025
0,020
0,015
0,010
0,005
830
820
Wavenumber (cm^{-1})
B
836 cm^{-1}
Band area (a.u.)
0.4
0.3
0.2
0.1
0.0
*
*
ND
D
D6U
C
836 cm^{-1}
r = - 0.788
p < 0.0001
Band area (a.u.)
0
200
400
600
Glycemia (mg/dL)
D
ROC curve of 836 cm^{-1}
Sensitivity (%)
100
50
0
AUC = 1
p = 0.0005
Sensitivity: 100 %
Specificity: 100 %
Specificity (%)
E
ROC curve of 836 cm^{-1}
AUC = 1
p = 0.0027
Sensitivity: 100 %
Specificity: 100 %
Sensitivity (%)
Specificity (%)

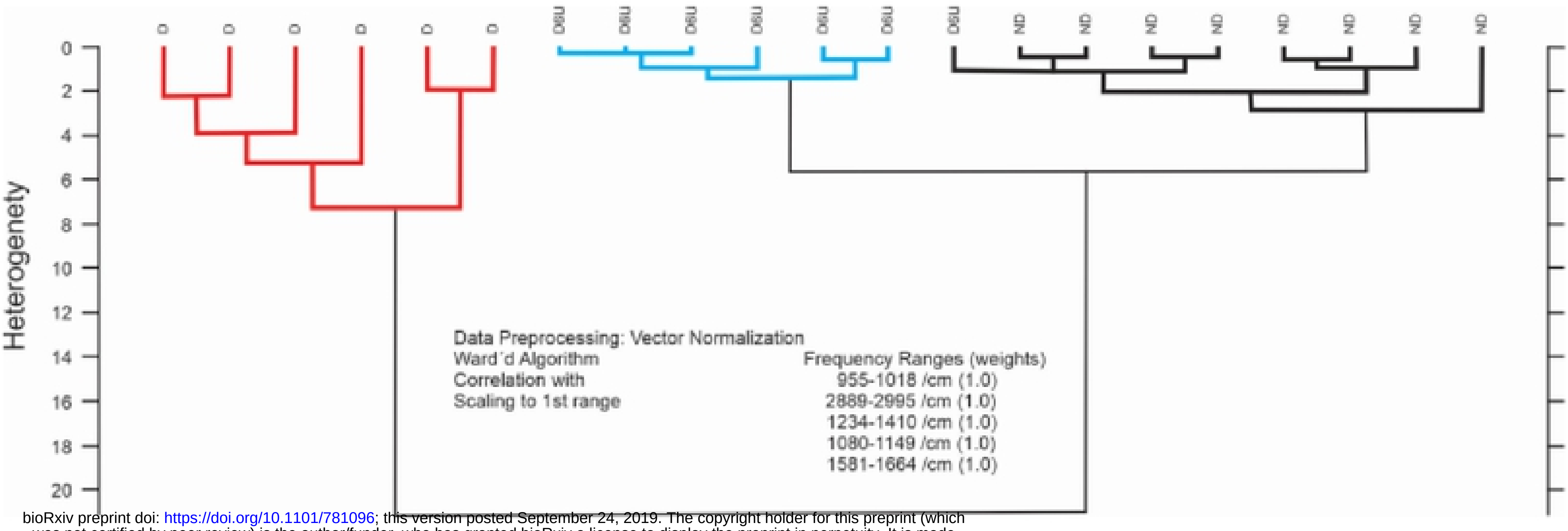
Figure 4.



	Validation set (n)					
	ND	D	D6U	Total (n)	Correct (n)	Accuracy (%)
ND	8	0	0	8	8	100
D	0	6	1	7	6	100
D6U	0	0	6	6	6	85.7

Correct Classification With Cross Validation		
(n)	Correct (n)	Accuracy (%)
21	20	**95.2**

Figure 5.


D
D
D
D
D
D
D6U
D6U
D6U
D6U
D6U
D6U
D6U
ND
ND
ND
ND
ND
ND
ND
ND
Heterogenety
0
2
4
6
8
10
12
14
16
18
20
Data Preprocessing: Vector Normalization
Ward´d Algorithm
Correlation with
Scaling to 1st range
Frequency Ranges (weights)
955-1018 /cm (1.0)
2889-2995 /cm (1.0)
1234-1410 /cm (1.0)
1080-1149 /cm (1.0)
1581-1664 /cm (1.0)