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Differential Molecular Signature of Human Saliva Using ATR-FTIR Spectroscopy for Chronic Kidney Disease Diagnosis

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The symptoms of chronic kidney disease (CKD) are often not specific or absent in the early stages of this illness. Therefore, there is a demand for developing low cost, non-invasive and highly accurate platforms for CKD diagnostics. We hypothesized that the level of specifics salivary components changes when CKD is emplace, which could be clinically used to discriminate CKD patients from healthy subjects. The present study aimed to compare salivary components between CKD patients and matched control subjects by using attenuated total reflection-Fourier Transform Infrared (ATR-FTIR) spectroscopy. The predictive power of salivary components was evaluated by receiver operating characteristic (ROC) curves. Several components were identified, and 4 of them showed different expression ($p<0.05$) between CKD and control subjects. Thiocyanate (SCN-, 2052 cm^{-1}) and phospholipids/carbohydrates (924 cm^{-1}) vibrational modes using original and second-derivative spectra by ATR-FTIR could potentially be used as salivary biomarkers to differentiate CKD than control subjects. The combination of original and second-derivative spectra by ATR-FTIR of 924 cm^{-1} vibrational modes could reach 92.8% sensitivity and 85.7% specificity for CKD detection. Despite, the limitation of our investigation, the acquired data indicates that salivary vibrational modes by ATR-FTIR platform should be further explored as an auxiliary diagnostic tool for CKD.

Key Words: human saliva, salivary biomarkers, renal disease, FTIR, hemodialysis, clinical spectroscopy.

Introduction

Chronic kidney disease (CKD) is defined by persistent urine abnormalities or impaired excretory renal function suggestive of a loss of functional nephrons (1). The global cost of CKD is over USD $1 trillion on end-stage renal disease care (2). Diabetes, hypertension, and glomerulonephritis are the leading causes of CKD (2). Currently, CKD is detected clinically by persistent increased urinary albumin excretion rate and for a decreased glomerular filtration rate (3). CKD is relatively silent for diagnosis, mainly in their early stages (4). Considering considerable loss of kidney function before symptoms become present, the dependence on the biomedical laboratory to establish the diagnosis of CKD is a relevant matter for research (4).

Saliva is a complex biological, body fluid derived from several tissue sources in various anatomic regions aside from just the proximate mouth. The majority of saliva derives from stimulation of specialized cells in salivary glands. In addition, Non-exocrine contributors involve host-derived entities, such as desquamated epithelial cells, intact and remnants of blood cells, and gingival crevicular fluid (6). In mammalians, about one-quarter of circulating inorganic nitrate (NO3–) derived from the diet. The salivary glands are the main responsible for delivering NO3-in saliva (5). Changes in salivary composition were demonstrated in CKD subjects. For example, high levels of creatinine, urea, sodium, potassium, chloride, salivary alpha-amylase (7), increase in salivary pH and buffer capacity (8), and higher levels of phosphate and albumin (9) were observed. Salivary inflammatory cytokines (TNF α, interleukin (IL) 1β, γ-interferon (γ-INF), IL-6, IL-8) were also in high concentration in saliva of CKD patients (10). On the other hand, the levels of immunoglobulin A, immunoglobulin G, and C reactive protein did not change in CKD subjects when compared with healthy subjects (11). There are many advantages of using saliva as a diagnostic fluid. Saliva collection is fast, easy, inexpensive and non-invasive (12).

The attenuated total reflection-Fourier Transform Infrared (ATR-FTIR) spectroscopy is a fast, highly sensitive and reproducible physicochemical analytical tool able to identify organic and inorganic molecules by infrared absorption. The ATR-FTIR spectroscopy makes possible to calculate several chemical constituents and specific proteins with high accuracy without requiring specific reagents (13). Here, we tested the hypothesis that specific vibrational modes of salivary components acquired by FTIR

spectroscopy could be used for discriminate patients with CKD from a group with CKD.

Material and Methods

The Institutional Ethics Committee of the Federal University of Uberlandia approved the study (IRB Number # 1.372.118). All methods to CKD diagnosis were performed following the relevant guidelines and regulations of National Kidney Foundation (NKF).

The present study included 14 patients diagnosed with CKD according to NKF, and 14 age- and gender-matched healthy control subjects with a negative history of kidney and systemic/oral disease (Table 1). Due to late stages of the disease, CKD subjects were undergoing hemodialysis. All participants had an oral examination before saliva collection.

Participants were requested abstain to eat, drink or brush their teeth 1 h before the saliva collection and not ingest alcoholic beverages 24h before saliva collection.

Infrared Spectroscopy

Samples were analyzed in 4000-400cm^{-1} region using infrared spectroscopy with attenuated total reflection accessory (FTIR-ATR Vertex 70, Bruker Optics, Reinstetten, Germany). Two µL of saliva were dried on ATR Crystal, and then the saliva spectra were acquired (13). Each sample and its background were recorded with 32 scans per analysis and 4 cm^{-1} resolution.

Spectral Data Analysis

The spectra were normalized by the vector method and adjusted to rubber band baseline correction. The obtained band areas were performed using Opus 6.5 software (Bruker Optics, Reinstetten, Germany). The original data were plotted in the Origin Pro 9.0 (OriginLab, Northampton, MA, USA) software to create the second derivative analysis. The second derivative was obtained by applying Savitzky-Golay algorithm with polynomial order 5 and 20 points of the window. The normalization of second derivatives was made by mean, and the peak heights indicated the intensity of each salivary component. Table 2 describes vibrational modes present in each band and the identification of the respective components found in the saliva (14,15).

In Vitro Correlation Analysis

Thiocyanate (G6639, Sigma, St. Louis, MO, USA) was diluted in 5 concentrations (0.78, 3.12, 12.5, 50 and 100 mg/mL). Two µL of each fluid was dried on ATR Crystal, and then the spectra of fluid with several SCN- concentrations were recorded. FTIR-based band area at 2063 cm^{-1} (2094-2014 cm^{-1}) parameters were compared with SCN- anions distributions by calculating Pearson's correlation coefficients.

Table 1. Demographic and seric laboratorial data of controls and patients with chronic kidney disease at the time of salivary sampling

	Cases	Controls
Age, years	55.2 ±11.2	54.3 ±14.0
Gender (M:F)	1:1	1:1
PTH, pg/mL	389 ± 323.5	-
Glicosis, mg/dL	155 ± 90.8	-
Ureia, mg/dL	142 ±24.9	-
Creatinine, mg/dL	9.0 ±3.6	-
Calcium, mg/dL	9.2 ±0.7	-
Phosphorus, mg/dL	5.0 ±1.5	-
Potassium, mEq/L	4.8 ±1.5	-
Sodium, mEq/L	138 ±2.6	-

Gender was reported as the proportion of male: female. All other values are reported as a mean ± standard variation. Number of CKD = 14 and controls = 14.

Table 2. Vibrational modes and identification of the respective salivary component

Band Frequency (cm^{-1})	Peak Frequency (cm^{-1})	Component Identification	Vibrational Mode
3680-2990	3364	Amide A (14)	Symmetric N-H stretching
2094-2020	2063	Thiocyanate anions (SCN-) (14)	C-N stretching
1730-1500	1635	Amide I (α-helix) (14)	C=O stretching
1490-1430	1452	Proteins (15)	Asymmetric CH_3 bending
1427-1368	1408	Proteins (15)	Symmetric CH_3 bending
1287-1220	1245	Amide III/ Phospholipids (14)	Asymmetric C-N stretching, PO^{2-} stretching
1190-1000	1075	Sugar moieties (14)	CH_2OH groups, C-O stretching & COH groups bending, symmetric PO^{2-} Stretching
1000-965	990	Polysaccharides (15)	OCH_3
955-905	924	Membrane lipids (phospholipids); Carbohydrates; (16)	CH3-N asymmetric stretching C-O, C-C stretching C-O-H, C-O-C Deformation

Statistical Analysis

The distributions of data were analyzed for D'Agostino-Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests. Then, data were analyzed using Student t-test ($p>0.05$) and Receiver Operating Characteristic (ROC) curve. The 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. Demographic, seric laboratorial data and salivary components were expressed as mean ± standard deviation (SD).

Results

Both groups included males and females subjects. CKD patients and matched healthy control subjects showed similar age (control 55.2 years old; range 33 to 74 years old and CKD 54.3 years old; range 36 to 74 years old). The etiologies of CKD among patients were diabetes mellitus (43% of the cases), chronic glomerulonephritis (43%) and hypertension (14%).

The infrared spectrum obtained in the saliva of control and CKD patients are represented in Figure 1. The comparison of these salivary spectra showed changes between control and CKD patients. The band area at 3364 cm^{-1} (3680-2990 cm^{-1}) was identified as amide A. CKD promoted decrease ($p<0.05$) in Amide A compared to control (Fig. 2A). The band area at 2063 cm^{-1} (2094-2014 cm^{-1}) indicates SCN- (SCN-, C-N stretching). This salivary component was strongly decreased ($p<0.05$) in CKD (Fig. 2B). The band area at 1635 cm^{-1} (1730-1500 cm^{-1}) determinates amide I. Amide I was not affected ($p>0.05$) by CKD (Fig. 3C). The band area at 1452 cm^{-1} (1490-1430 cm^{-1}) is identified as asymmetric CH3 bending. The asymmetric CH3 bending component was increased ($p<0.05$) in the saliva of CKD patients compared with controls (Fig. 3D). The bands at 1408 cm^{-1} (1427-1368 cm^{-1}) and 1245 cm^{-1} (1287-1220 cm^{-1}) are identified as symmetric CH3 bending and amide III/ phospholipids, respectively. Both salivary components were unchanged ($p>0.05$) (Fig.3E-F) in CKD compared with control subjects. The band's area at 1075 cm^{-1} (1190-1000 cm^{-1}) and 990 cm^{-1} (1000-965 cm^{-1}) are characterized as glycosylated proteins and polysaccharides, respectively. The levels of both components were unchanged ($p>0.05$)

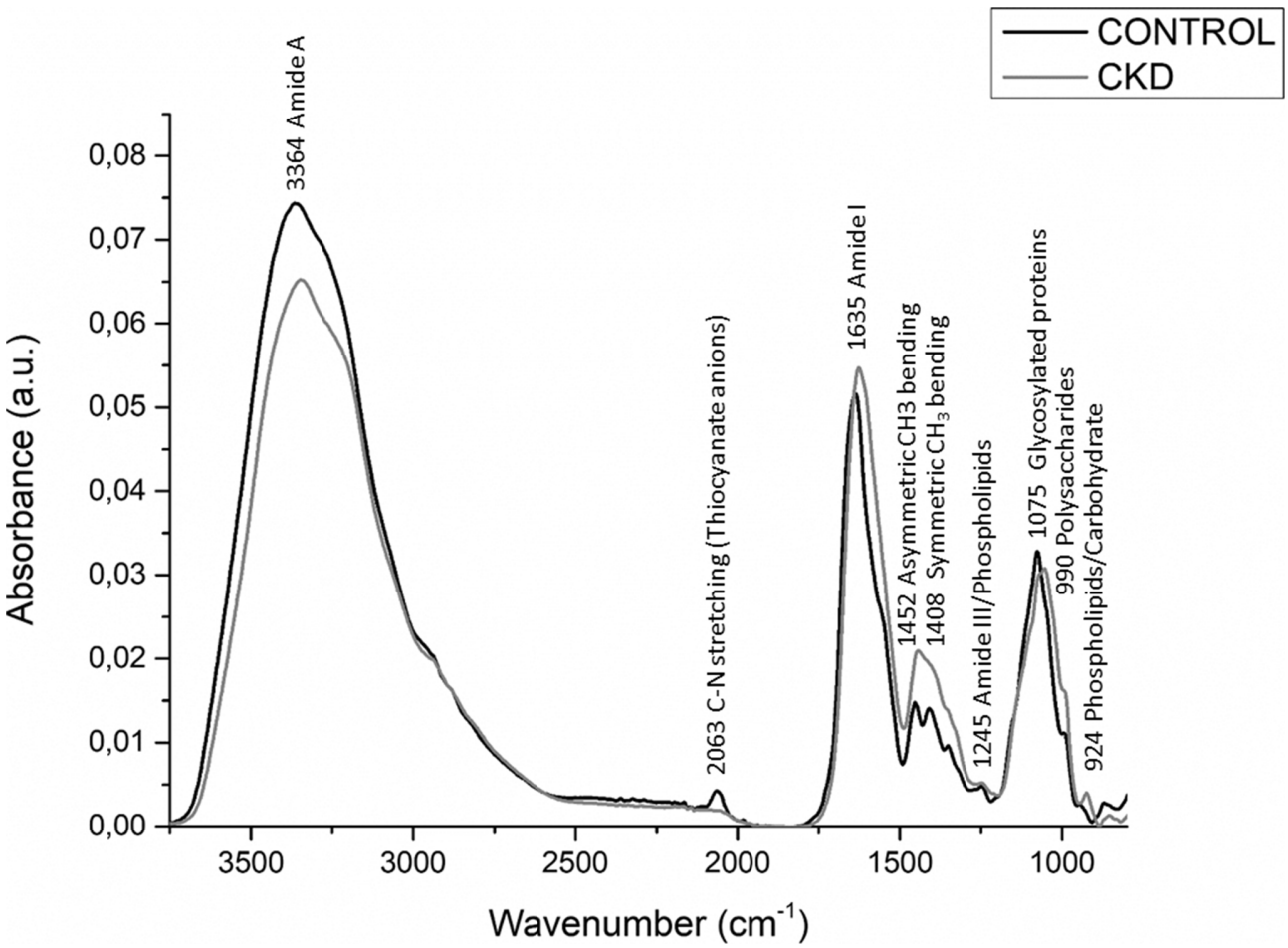

Figure 1. Comparison of the average FTIR spectrum in control subjects and Chronic Kidney Disease (CKD) patients.

(Fig. 3G-H) in CKD patients compared to control subjects. The band at 924 cm^{-1} (955-905 cm^{-1}) was indicated as phospholipids/carbohydrates (Fig. 3I). These salivary components at 924 cm^{-1} was increased ($p<0.05$) in CKD patients when compared to control subjects.

The diagnostic performance of these potential markers was further evaluated for detection of CKD. The ROC curve was performed to predict a threshold value of salivary Amide A (3364 cm^{-1}), SCN- (2063 cm^{-1}), asymmetric CH3 bending (1452 cm^{-1}) and phospholipids/carbohydrate (924 cm^{-1}) that could be used for diagnosis of CKD, and is shown in Figure 3. The area under the curve (AUC) of salivary Amide A (3364 cm^{-1}) was 0.73 ($p=0.03$), and the best diagnostic threshold value was 21.82 with a sensitivity of 71.4% and specificity of 78.6% (Fig. 3.A). The area under the curve (AUC) of SCN- (2063 cm^{-1}) was 0.82 ($p=0.003$), and the best diagnostic threshold value was 0.016 with a sensitivity and specificity of 78.6% (Fig. 3.B.). The area under the curve (AUC) of asymmetric CH3 bending (1452 cm^{-1}) was 0.79 ($p=0.007$), and the best diagnostic threshold value was 0.173 with a sensitivity and specificity of 71.4% (Fig. 3.C.). The area under the curve (AUC) of salivary phospholipids/carbohydrate (924 cm^{-1}) was 0.74 ($p=0.02$) and the best diagnostic threshold value was 0.0445 with a sensitivity

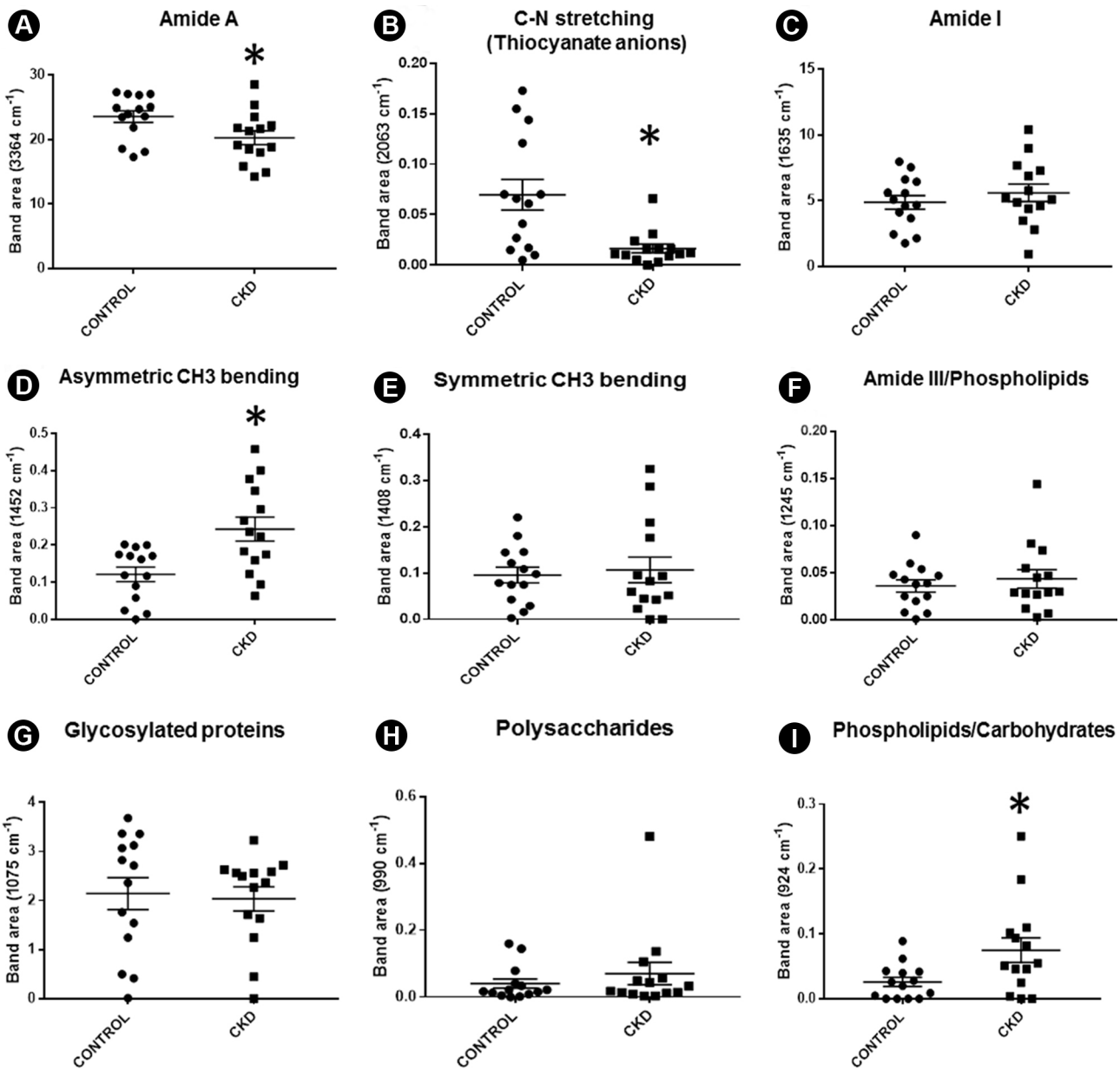

Figure 2. Level of Amide A(A), SCN- (B), Amide I (C), Asymmetric CH3 bending (D), Symmetric CH3 bending (E), Amide III/Phospholipids (F), Glycosylated proteins (G), Polysaccharides (H) and Phospholipids/Carbohydrates (I) in saliva of control subjects and CKD patients. Results are mean ± SD. *$p<0.05$ vs. control. Unpaired t-test. n = 14 for patients with CKD, n = 14 for control subjects.

of 71.4% and specificity of 85.7% (Fig. 3.D).

We also carried out the second derivative of ATR-FTIR spectrum of Amide A (3450 cm^{-1} to 3390 cm^{-1}) to amplify the spectral variations of saliva (Fig. 4. A-C). Band identified the level of Amide A at 3433 $cm^{-1,}$ and this vibrational mode was reduced ($p<0.05$) in the saliva of CKD patients compared to control subjects (Fig. 4.D). The area under the curve (AUC) of second derivative salivary Amide A (3433 cm^{-1}) was 0.75 (p=0.021), and the best diagnostic threshold value was 0.016 with a sensitivity of 71.4% and specificity of 78.6% (Fig. 4.G). The level of 3410 cm^{-1} vibrational mode was reduced ($p<0.05$) in the saliva of CKD patients compared to control subjects (Fig. 4.E). Although the similar sensitivity and specificity, the AUC of salivary Amide A (3410 cm^{-1}) in the second derivative was 0,79 (p=0.007) with a diagnostic threshold value of 0.008. The level of Amide A was also identified by a band at 3396 $cm^{-1,}$ and this vibrational mode was also reduced ($p<0.05$) in the saliva of CKD patients compared to control subjects (Fig. 4.F). The area under the curve (AUC) of second derivative salivary at 3433 cm^{-1} was 0.77 (p=0.013), and the best diagnostic threshold value was 0.01 with a sensitivity of 78.6% and specificity of 764.29% (Fig. 4.I).

We also carried out the second derivative of ATR-FTIR spectrum of C-N stretching (SCN-; 2080 cm^{-1} to 3390 cm^{-1}) and phospholipids/carbohydrates (950 cm^{-1} to 918 cm^{-1}) to amplify the spectra of saliva (Fig. 5.A and 5.B). The band at 2052 cm^{-1} was decreased ($p<0.05$) in the saliva of CKD as compared to controls (Fig. 5.C). The area under the curve (AUC) of second derivative salivary SCN-was 0.76 (p=0.016) and the best diagnostic threshold value was 0.01 with a sensitivity of 85.7% and specificity of 71.4% (Fig. 5.E). The phospholipids/carbohydrates at 924 cm^{-1} was increased ($p<0.05$) in saliva CKD as compared to controls (Fig. 5.D). The area under the curve (AUC) of second derivative salivary phospholipids/carbohydrates

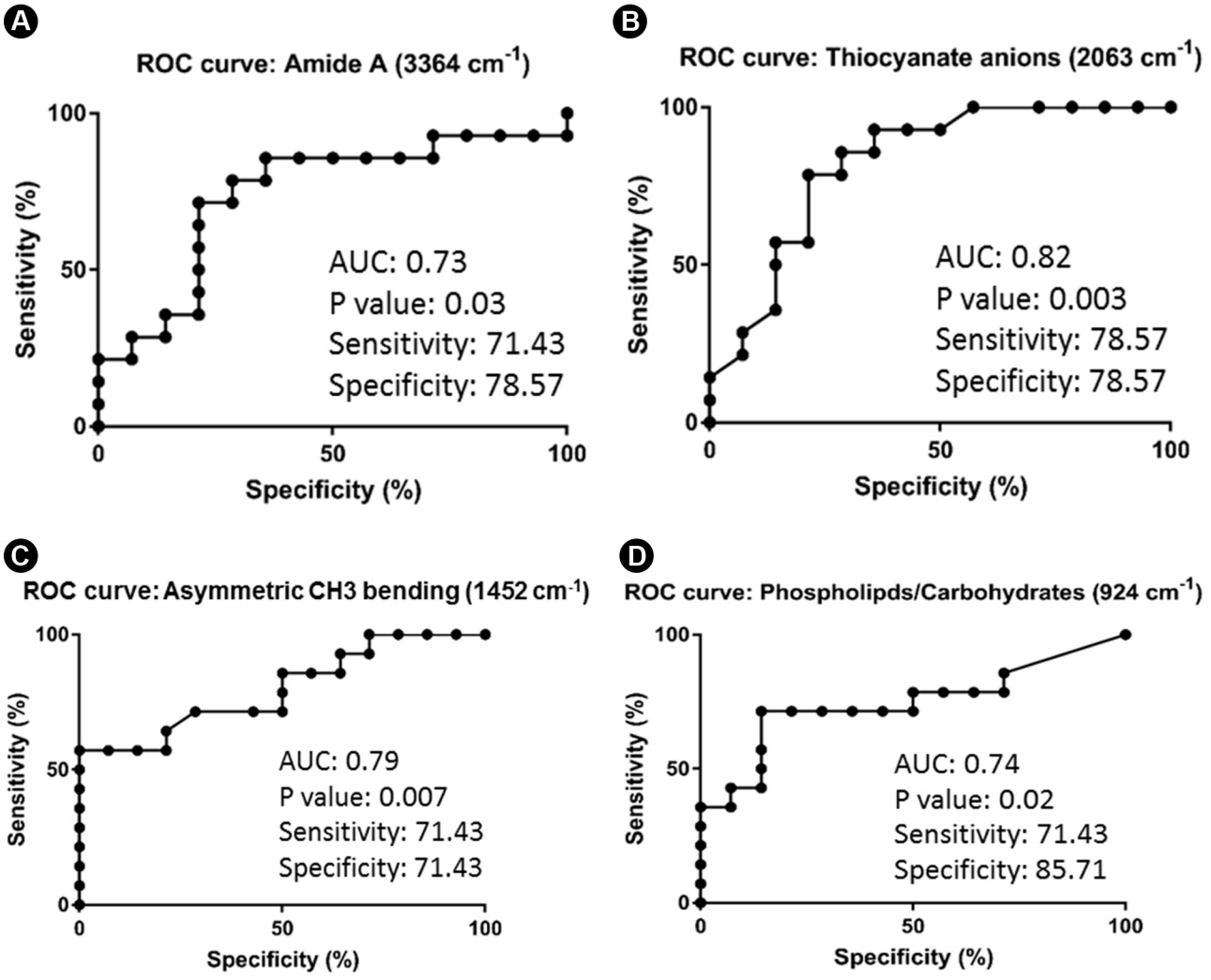

Figure 3. ROC curve for salivary Amide A(A), SCN- (B), Asymmetric CH3 bending (C) and Phospholipids/Carbohydrates (D) control subjects and CKD patients. *p<0.05 vs. control. Unpaired t-test. n = 14 for patients with CKD, n = 14 for control subjects.

at 924 cm^{-1} was 0.88 (p=0.0005), and the best diagnostic threshold value was 0.003 with a sensitivity of 92.9% and specificity of 71.4% (Fig. 5.F).

The infrared spectrum obtained in 5 concentrations of SCN- (0.78, 3.12, 12.5, 50 and 100 mg/ml) are represented in Figure 5A. The comparison of these salivary spectra showed changes in each fluid with increasing concentration of SCN-. The band area at 2063 cm^{-1} (2094-2014 cm^{-1}) indicates SCN- by FTIR analysis correlate with changes in SCN- concentration (P value: 0.04 and Pearson r: 0.879).

Discussion

The presented data support the hypothesis that two vibrational modes of saliva may discriminate patients with CKD and healthy controls. Here we indicated potential new salivary ATR-FTIR biomarkers for CKD diagnosis or CKD screening. SCN- (2052 cm^{-1}) and phospholipids/carbohydrates (924 cm^{-1}) vibrational modes using original and second-derivative spectra by ATR-FTIR could potentially be used as salivary biomarkers to differentiate CKD patients than matched control subjects. Although several studies showed the measurement of salivary creatinine and urea levels as a biomarker of CKD (7,17), the use of ATR-FTIR technology may be a more rapid, cost-effective and convenient salivary diagnostic platform for CKD. The discriminatory power for CKD of these salivary vibrational modes can reach AUC of 0.88 and high levels of specificity (85.7%) and sensitivity (92.8%), suggesting that salivary

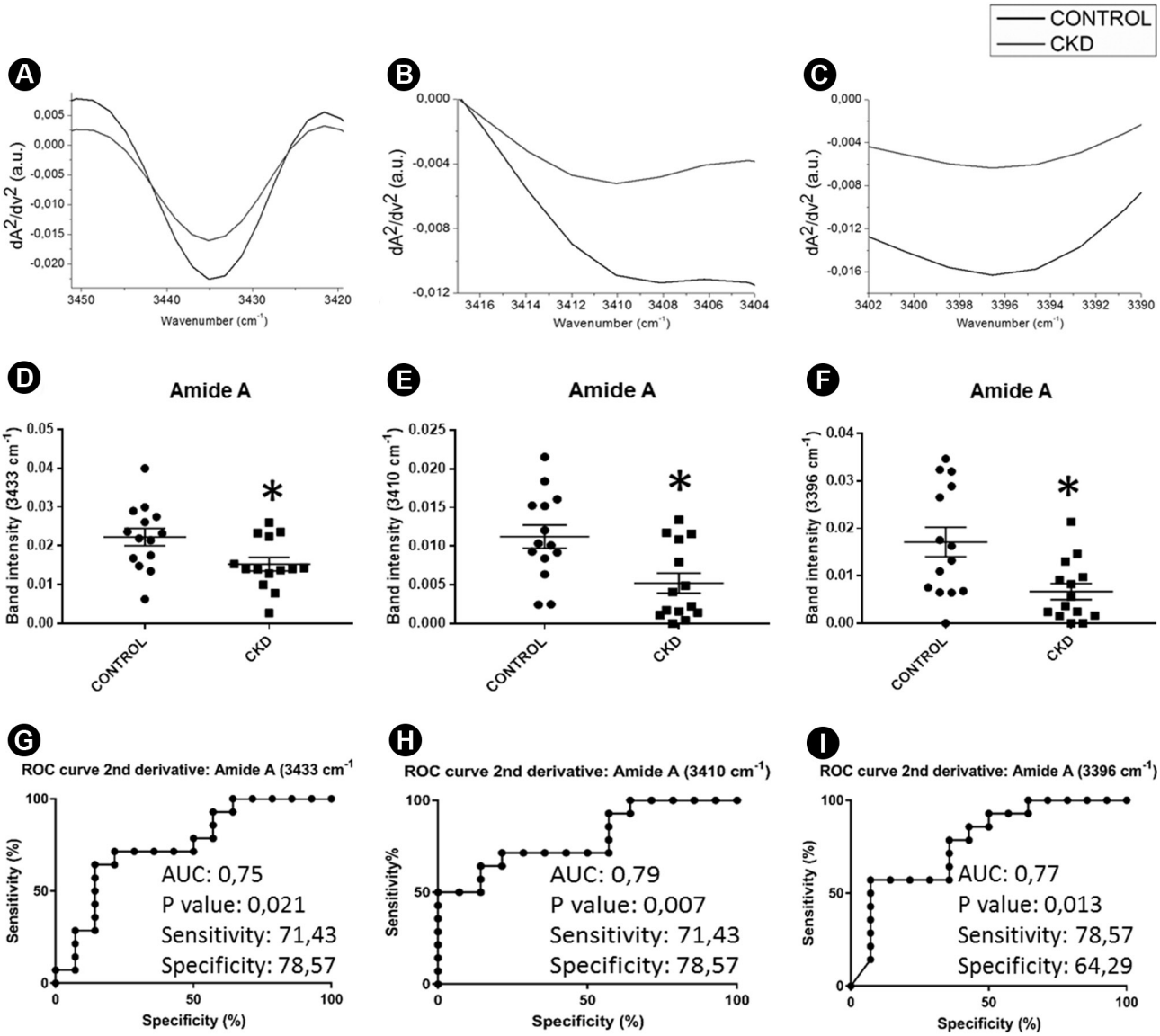

Figure 4. Comparison of the second-derivative spectra average by ATR-FTIR, levels and ROC curve analysis of three vibrational modes of Amide A in control subjects and Chronic Kidney Disease (CKD) patients. *p<0.05 vs. control. Unpaired t-test. n = 14 for patients with CKD, n = 14 for control subjects.

vibrational modes had a high capacity to discriminate patients with chronic kidney disease from healthy people.

Salivary creatinine and urea levels have been considered as effective clinical values in CKD diagnosis with good diagnostic accuracy (17). However, due to the high costs involved in performing the salivary creatinine diagnosis; the salivary level of SCN- (2052 cm^{-1}) of original ATR-FTIR spectra and salivary phospholipids/carbohydrates (924 cm^{-1}) levels of second derivative ATR-FTIR spectra may be a more convenient and cost-effective diagnostic platform for CKD. Both ATR-FTIR biomarkers are a compatible surrogate for salivary creatinine and serum for diagnosis of CKD.

SCN- is a small, acidic pseudohalide thiolate that is ubiquitously found in the extracellular fluids, including plasma and saliva. SCN- is synthesized from cyanide by mitochondrial rhodanese or enters the blood from the diet (18). Chronic renal failure patients undergoing hemodialysis showed a reduction in SCN- formation, indicating reduction cyanide detoxication capability due to the parallel regulation of rhodanese (18). Despite this,

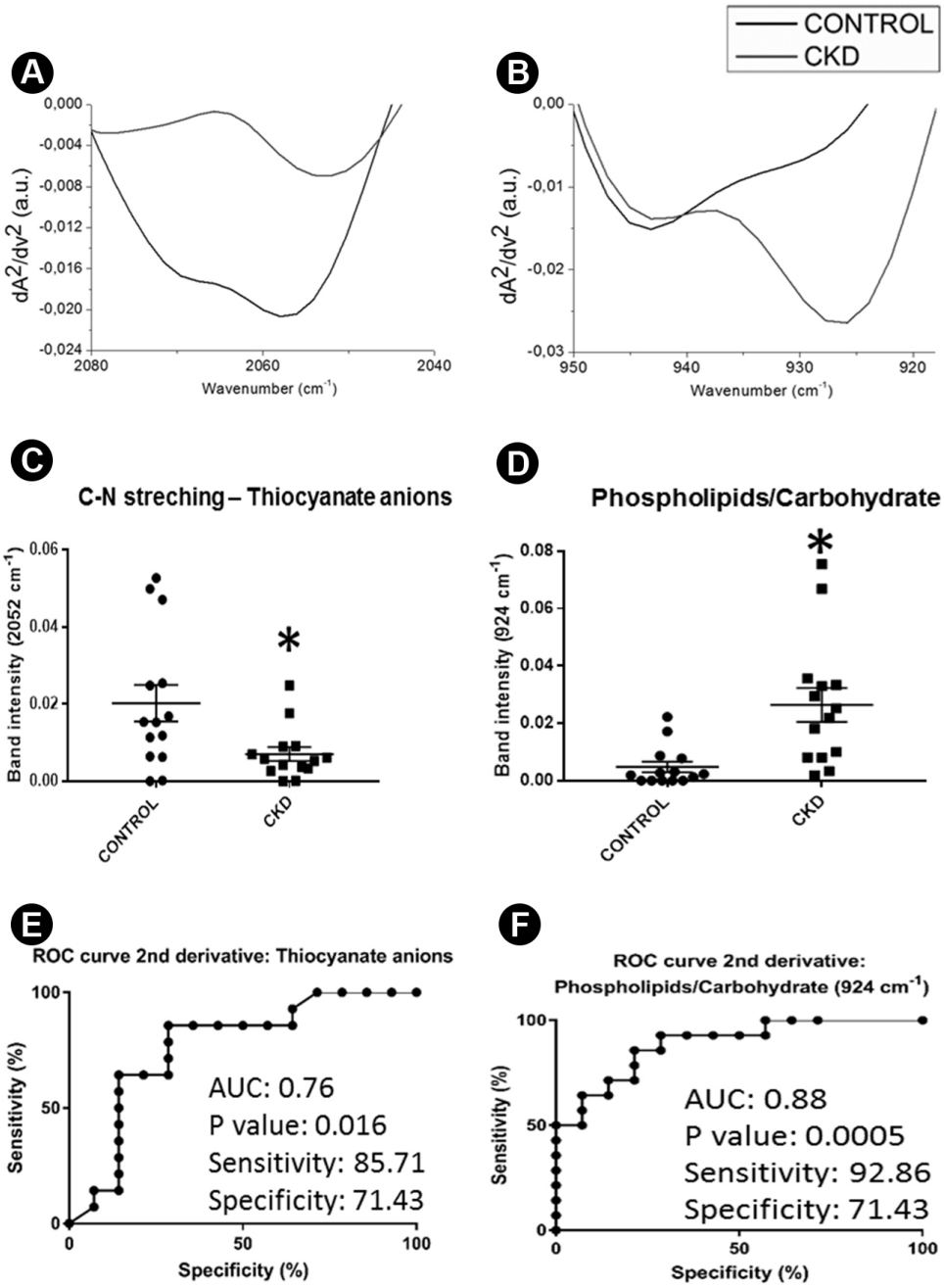

Figure 5. Comparison of the second-derivative spectra average by ATR-FTIR, levels, and ROC curve analysis of SCN-and Phospholipids/Carbohydrates vibrational modes in control subjects and Chronic Kidney Disease (CKD) patients. *p <0.05 vs. control. Unpaired t-test. n = 14 for patients with CKD, n = 14 for control subjects.

the plasma SCN- concentration is increased in chronic renal failure patients due to the reduction in kidney elimination of SCN- is pivotal to change plasma SCN- concentration (19). The SCN- in saliva ranges from 0.5 to 3 mM and making saliva the most SCN-rich matrix in the body (18). It suggests that the SCN- salivary concentration depends more on the active transcellular transport of SCN- in acinar cells than on the SCN- plasma concentration. SCN- enters secretory epithelia from the blood by sodium-iodide symporter (NIS) and its carrier into the acinar lumen by cystic fibrosis transmembrane conductance regulator (CFTR) transporter (18). This finding predicts that reduction in NIS and CFTR transporters in acinar cells of salivary glands can reduce the SCN- salivary concentration. Another hypothesis to explain the reduction of SCN- salivary concentration is related to catalyzes the oxidation of SCN- in the presence of hydrogen peroxide (H_2O_2) and glandular peroxidase to generate hypothiocyanite (OSCN). It is corroborated by the increase in peroxidase and reactive oxygen species in acinar cells of salivary glands described in rats with chronic kidney disease induced by 5/6 nephrectomy (20). Besides, the reduction of salivary OSCN, which inhibits bacterial growth, is compatible with an increase in gingival inflammation and plaque of chronic kidney disease patients.

The high levels of salivary phospholipids/carbohydrates verified at 924 cm^{-1} in second derivative ATR-FTIR spectra indicate a wavenumber with higher spectroscopy efficiency to discriminate CKD. Considering that this wavenumber indicates phospholipids and carbohydrates, the pathophysiological effects could be inadequately discussed. Although the precise molecule cannot be defined, the potential diagnostic of 924 cm^{-1} in second derivative ATR-FTIR spectra is unequivocal.

One limitation of this study is the sample size. While not statistically significant, some ROC curves showed magnitudes that conceivably may have achieved

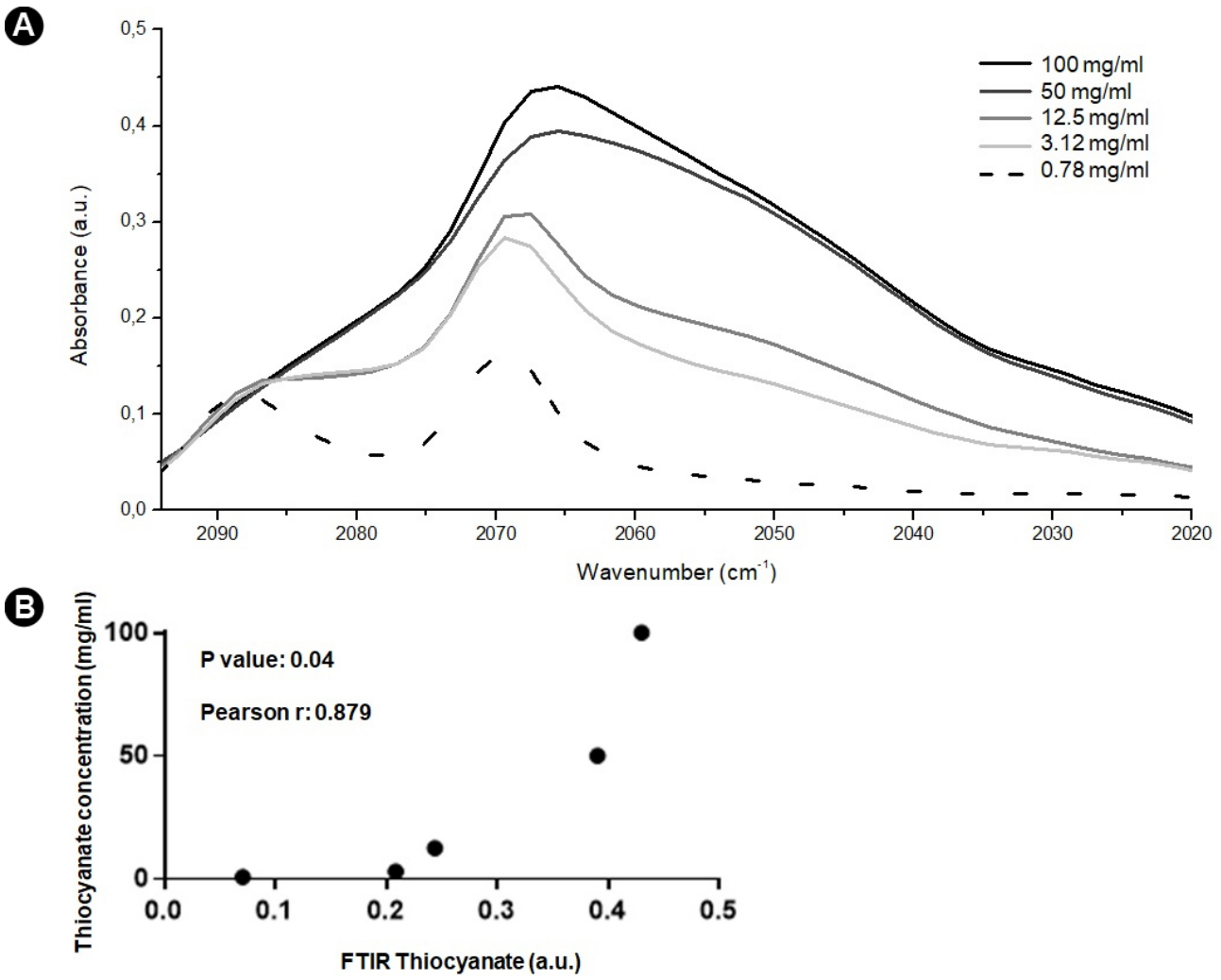

Figure 6. Comparison of the average FTIR spectrum obtained from 5 concentrations of SCN- (0.78, 3.12, 12.5, 50 and 100 mg/mL, A) and correlation of ATR-FTIR analysis and SCN- concentration. Pearson correlation.

significance with more significant sample. Patients in early and moderate stage CKD must be included in further studies to improve ATR-FTIR as a diagnostic platform for this disease. Another limitation of the present study is the not intentional inclusion of CKD patients in stages 4 and 5. However, this condition is representative of CKD patients in our environment due to health care factors. Further studies are needed to assess the diagnostic performance of these spectral FTIR biomarkers in saliva in other renal pathologies populations., as nephrotic syndromes, interstitial tubular diseases, renal papilla necrosis, and glomerulonephritis.

In conclusion, our data show that 2052 cm^{-1} and 924 cm^{-1} ATR-FTIR vibrational modes were able to discriminate CKD patients from healthy subjects. In addition, the combination of original and second-derivative spectra by ATR-FTIR of 924 cm^{-1} vibrational modes reached 92.8% sensitivity and 85.7% of specificity for the detection of CKD. Both vibrational modes using original and second-derivative spectra by ATR-FTIR could potentially be used as salivary biomarkers to differentiate CKD than matched control subjects.

Resumo

Os sintomas da doença renal crônica (DRC) são frequentemente inespecíficos ou ausentes nos estágios iniciais desta doença. Desta forma, existe uma demanda para o desenvolvimento de plataformas com baixo custo, não-invasivas e com alta acurácia para o diagnóstico da DRC. Nós hipotetizamos que o nível dos componentes salivares se alteram pela DRC, o que pode ser clinicamente utilizado para discriminar pacientes portadores de DRC de indivíduos controles. O objetivo deste estudo foi comparar componentes salivares entre pacientes portadores de DRC e sujeitos controles utilizando um sistema de reflectância total atenuada com espectroscopia infravermelho com transformada em Fourier (ATR-FTIR). O poder preditivo dos componentes salivares foi avaliado pela curva característica de operação do receptor (ROC). Diversos componentes salivares foram identificados e 4 destes apresentaram diferença na expressão ($p<0,05$) entre DRC e sujeitos controles. O modos vibracionais do tiocianato (2052 cm^{-1}) e de fosfolipídeos/carbohidratos (924 cm^{-1}) utilizando espectros originais e da segunda-derivada pelo ATR-FTIR podem potencialmente ser utilizados como biomarcadores salivares para discriminar a DRC de sujeitos controles. A combinação dos espectros originais e da segunda-derivada pelo ATR-FTIR do modo vibracional 924 cm^{-1} pode apresentar sensibilidade de 92.8% e especificidade de 85.7% para a detecção da DRC. Este estudo indicou que modos vibracionais da saliva pela plataforma ATR-FTIR podem ser uma ferramenta auxiliar no diagnóstico da DRC.

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