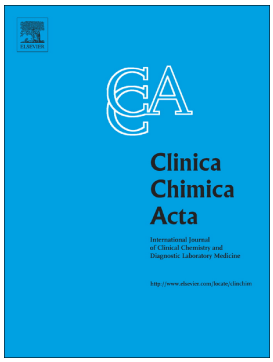

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Identification of salivary peptidomic biomarkers in chronic kidney disease patients undergoing haemodialysis

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Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate; CHCA, alpha-cyano-4-hydroxycinnamic acid; FDR, false discovery rate; KNN, k-nearest-neighbour; S WCX, weak cation exchange; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; 2nano-UPLC/ESI–MS/MS, nano-ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry; aPRP 1/2, acidic proline-rich phosphoprotein 1/2; SMR 3B, submaxillary gland androgen-regulated protein 3B; DMFT, decayed, missing, and filled teeth

ABSTRACT

Background

Chronic kidney disease (CKD) is a worldwide public health problem. To detect discriminating salivary peptide profiles between CKD patients undergoing haemodialysis (HD) and healthy controls (HCs) and to screen candidate biomarkers for CKD, we preliminarily explored the diagnostic potentiality of saliva.

Methods

Saliva samples from 30 CKD HD patients and 35 HCs were analysed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) combined with weak cation exchange magnetic beads. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was performed to identify the potential biomarkers.

Results

The HD group showed higher salivary pH. We chose 3 significantly different ($p < 0.01$) peptides to establish an effective discrimination model which was validated by a decision tree and ROC curve. Statistically significant correlations ($p < 0.05$) between the candidate salivary peptidomic biomarkers and serum parameters were found. Eight differentially expressed peptides were identified as segments of Histatin-1, Mucin-7, salivary acidic proline-rich phosphoprotein 1/2 (aPRP 1/2), submaxillary gland androgen-regulated protein 3B, and Histatin-3.

Conclusions

Our findings substantiate the pivotal role of peptidomic methods in identifying biomarkers

for CKD. Histatin-1, Mucin-7 and aPRP 1/2 are expected to be candidate salivary biomarkers of CKD HD patients. Furthermore, saliva might be a promising non-invasive diagnostic biofluid.

Keywords: MALDI-TOF MS, UPLC-MS/MS, Salivary peptidomic biomarkers, Chronic kidney disease (CKD), Haemodialysis

1. Introduction

Chronic kidney disease (CKD) is a general concept resulting from many heterogeneous disorders that irreversibly affect the kidney. Current international guidelines define CKD as abnormalities in kidney structure or function that are present for at least 3 months, with implications for health [1]. The diagnostic criteria for CKD include a glomerular filtration rate (GFR) of less than 60 ml/min per 1.73 m², or markers of kidney damage, or both for a duration of at least 3 months, regardless of the underlying cause [2]. When GFR is less than 15 ml/min per 1.73 m², replacement therapy (in the form of dialysis or transplantation) is needed to sustain life. CKD was reported to have an estimated prevalence of 11% worldwide [3] and 10.8% in China [4]. Adverse outcomes of CKD include not only progression to kidney failure but also many complications of reduced kidney function and increased risk of cardiovascular disease and other chronic diseases [5]. CKD ranked 19th in the list of global causes of years of life lost in 2013 [6]. The increasing prevalence, considerable morbidity and mortality, high medical costs, and poor treatment outcomes constitute a major public health burden, which presents a significant challenge to global health policy [7]. Therefore, the prompt detection, diagnosis and prevention of CKD in an early stage are extremely imperative.

The diagnosis of nephropathy can be determined by clinical symptoms, laboratory examinations, or image analysis. Biomarkers derived from serum and urine play an important role in the diagnosis and assessment of the risk of CKD. Although GFR is currently considered to be the best indicator of CKD [8], the accuracy of diagnosis is affected by the intricate parameters and complex evaluation methods, including the adoption of different

formulas. Moreover, urine collection is inconvenient for some patients, and blood serum analysis is often unsuitable due to the invasive procedure and the associated risk of infection, which cause nervousness among patients [9]. Therefore, a non-invasive diagnostic medium that provides an accurate evaluation of disease status is necessary.

Saliva, a multi-constituent biological fluid secreted by salivary glands, is considered a filtrate of the blood. Therefore, saliva can reflect the physiological status of the body, as confirmed in the detection of head and neck carcinoma, breast cancers, gastric cancers and other systemic diseases [10]. Some changes in saliva composition, flow rate and buffering capacity have been detected in renal diseases [11,12]. Previous studies have found statistically significant correlations between serum and salivary parameters of patients with CKD [13]. With the rapid development of proteomic technologies, more than 3,000 proteins with different functions have been identified in saliva thus far [14]. Some of these proteins may contribute to the diagnosis and monitoring of many diseases at the protein level [15]. Recently, developed proteomics techniques offer new options for the isolation and identification of proteins and peptides with clinical relevance. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), an emerging sensitive proteomic technique, allows the presence, expression levels, and posttranslational modifications of proteins in the proteome to be detected in minute detail [15]. Although this method has been widely applied to studies of serum and urine in the field of nephrology in recent years [16-18], few reports of salivary proteomics associated with CKD using this approach are available, and the peptidomic biomarker derived from the saliva of CKD patients has yet to be elucidated. In this study, MALDI-TOF MS was applied to analyse the

salivary peptides of CKD patients in combination with weak cation-exchange (WCX) magnetic beads (MB); subsequently, ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was performed to identify the proteins that might be the source of these peptides as candidate proteomic biomarkers.

2. Materials and methods

2.1. Ethics statement

This study received ethical approval by the Institutional Review Board (IRB) of Peking University School of Stomatology and was performed in accordance with the Declaration of Helsinki. All of the participants signed written informed consent before participation.

2.2. Subjects

Thirty CKD HD patients were recruited at the Renal Department of Internal Medicine, First Affiliated Hospital of PLA General Hospital in November 2016. Thirty-five HCs were selected from the pool of students and staff of Peking University Hospital of Stomatology in the same period.

The inclusion criteria were as follows: (1) 18-70 years old; (2) HD group with an estimated GFR < 15 ml/min per 1.73 m², clinically diagnosed according to the international classification criteria [2], and on haemodialysis for more than 1 year with no comorbid medical conditions such as diabetes mellitus and primary hypertension; (3) HC group with an estimated GFR ≥ 90 ml/min per 1.73 m², normal renal function as identified by urine and serum biochemistry, and no history of kidney disease and other chronic debilitating illnesses.

The following individuals were excluded: subjects with acute or chronic infections and malignant tumours; patients with a recent history of taking any medication that could affect the saliva features.

2.3. Saliva collection and processing

All subjects rinsed their mouths with clean water after breakfast followed by a 10 min interval before collecting 1.5 ml of unstimulated whole saliva samples. The saliva samples were immediately placed on ice. The supernatants were collected after centrifugation at 10,000 × *g* for 10 min at 4 °C. To inhibit protease activity, 1 mM ethylene diamine tetraacetic acid (Sigma) and 1 mM phenylmethyl sulfonyl fluoride (Sigma) were added. The Lowry method and an ELx808 Protein Assay (BioTek) were used to measure protein concentrations. Then, the supernatants were stored at -80 °C.

The salivary pH values were measured with pH strips (Spezialindikator, Merck, Darmstadt, Germany) according to the manufacturer's instructions [19].

2.4. Serum parameters

Data on serum creatinine and blood urea were obtained from the routine tests of blood that all participants underwent before the study commenced.

2.5. WCX fractionation and MALDI-TOF MS

WCX magnetic beads (Bioyong Techl were used to fractionate saliva samples. The samples were purified and isolated as follows: (1) 10 μL of salivary sample, 150 μl of MB-WCX binding solution, and 20 μl of beads were gently mixed and incubated for 5 min. (2) The tubes were placed on the MB separation device (Bioyong Tech) for 1 min, with the

beads adsorbing onto the tube wall, and then, the supernatant was removed. (3) The beads were washed and mixed with 150 μl of MB washing solution for 2 min, and the tubes were then maintained on the separation device for 1 min; then, this step was repeated to remove all the supernatants. (4) 10 μl of MB elution solution was added to the beads attached to the tube wall in the separation device for 2 min. (5) The clear supernatant was moved into a new tube, and the peptides were immediately analysed on a ClinTOF instrument (Bioyong Tech) or stored at -20 °C.

The matrix solution was 8 mg/ml alpha-cyano-4-hydroxycinnamic acid (CHCA) dissolved in acetonitrile, TFA and deionized water. One microliter of purified peptide solution was spotted onto a MALDI-TOF MS target. The sample was then dried at room temperature, covered with 1 μl of matrix solution and subsequently dried again. MALDI-TOF MS was conducted using a ClinTOF instrument (Bioyong Tech). We employed a 3-peptide mixture (monoisotopic molecular weights of 1533.8582, 2465.1989, and 5730.6087 Da; Sigma product numbers P2613, A8346, and I6279, respectively) for analysis calibration. We obtained the profile spectra from an average of 400 laser shots per sample. A mass range of 1000–10,000 Da was acquired. Each sample of saliva was analysed 3 times to acquire the mean values of the intensities and masses of each peak. This methodology has been successfully applied in previous published studies by our research group, indicating its reliability [20,21].

2.6. Data processing

By analysing the spectra obtained from the saliva samples using Bio Explorer (Bioyong

Tech), the mean relative peak intensities were determined; then, chemical and electrical noises were subtracted, the spectra were normalized by applying the total ion current, and peak mass-to-charge ratio (*m/z*) values and intensities were determined in the mass range of 1000–10,000 Da. A signal-to-noise (S/N) ratio > 5 was required. A mass shift of no more than 0.1% was determined to align the spectra. The peak area was analysed for quantitative standardization.

We employed the k-nearest-neighbour (KNN) algorithm to establish the best pattern of the discrimination model for CKD. A decision tree classification using the intensities of the peptides was constructed with the aim of validating the differentiation effectiveness of KNN model, and providing thresholds of the candidate clinical predictor for CKD.

2.7. Identification of potential peptide biomarkers by nano-ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (nano-UPLC/ESI–MS/MS)

The supernatants were processed as previously described, and the samples were then centrifuged at 10,000 × *g* for 20 min at 4 °C to remove the impurities. The supernatants were purified by a 0.22-μm filter before LC-MS/MS. An ACQUITY nano-UPLC system (Waters) was used to separate the sample. The separation was carried on an equilibrated BEH nano ACquity column 100 ×100 mm. The flow rate was set at 400 nL/min. A Q-Exactive mass spectrometer (Thermo Fisher Scientific) was used for tandem mass spectrometry (MS/MS). The voltage of the ion source was 3.5 kV, and the analysis time was set to 120 min. Higher-energy collision dissociation was used to fragment the peptides. The *m/z* values of the

peptide fragments were acquired by converging 20 fragmented fingerprints after each single full scan. A scan resolution of 70,000 was obtained at *m/z* 200 with MS and of 17,500 at *m/z* 200 with MS/MS. Initial screening was performed after the MS/MS figure was input into the PD software (Proteome Discoverer 1.4, Thermo Fisher Scientific).

2.8. Identification of candidate saliva biomarkers

With the results of the initial screening above, we searched the mass spectrum using Mascot software (ver 2.3.2) to predict the protein source of these peptides. Database NCBInr 20120419 (17893860 sequences; 6141683785 residues), and the Percolator method (false discovery rate [FDR] ≤ 1%; accuracy of peptide tol: 20 ppm) was applied for analysis.

2.9. Statistical analysis

The spectrum data were analysed using the BioExplorer statistical package. According to the results of normality tests, Student's *t*-test or the Wilcoxon rank sum test was used to analyse significant differences in peptide levels among saliva samples between the 2 groups ($p < 0.01$).

The differences in age and gender were compared using the *t*-test and the X^2 test, respectively. The salivary pH values were compared using the Mann-Whitney U test ($p < 0.01$). Pearson's correlation coefficient was used to calculate correlations between peak intensities of salivary peptidomic biomarkers (normally distributed after logarithmic transformation) and levels of serum parameters (normally distributed) ($p < 0.05$). These statistical analyses were performed using SPSS 20.0 software (IBM). Decision tree

classification was performed with the Biomarker Patterns Software 5.0.2 (Ciphergen Biosystems Inc.). In addition, the receiver operating characteristic (ROC) curve was analysed to estimate diagnostic efficacy of the KNN model.

3. Results

3.1. Sample information

In total, 35 healthy individuals (average age of 44 ± 11 years; 15 males and 20 females) were selected for the HC group, and 30 patients with CKD who were undergoing haemodialysis (average age of 49 ± 13 y; 15 males and 15 females) were recruited as the HD group. Details of these participants are shown in Supplementary Table 1. There were no significant differences between the 2 groups in age or gender. A total of 65 saliva samples were obtained.

3.2. Salivary pH values

The HD group showed higher pH values (mean ± S.D.: 8.20 ± 0.44) than the HC group did (mean ± S.D.: 7.50 ± 0.37) ($p < 0.01$). Details of pH values are shown in Supplementary Table 1.

3.3. Salivary peptide profiles

To compare the differences between the 2 groups, the entire mass spectra of the peptides from all the samples were analysed by MALDI-TOF MS (Fig. 1). Saliva peptidome fingerprint peaks were quantified based on the maximum intensity within a particular *m/z*

range. A total of 199 peptide mass peaks were detected in the 2 groups, as shown in Supplementary Table 2. The peak intensities differed significantly for 45 peptides ($p < 0.01$). Thirty-seven of these peptides were eliminated because of the lower relative average intensities and occurrence frequencies. The average intensities for the remaining eight peptide peaks (experimental *m/z* values: 2474.9, 2618.5, 2255.4, 2343.7, 2113.5, 1278.9, 1226.7 and 1307.0 Da) are listed in Supplementary Table 3.

The HD group showed higher mass peaks for peptides of 2474.9, 2618.5, 2255.4, 2343.7 and 2113.5 Da and lower mass peaks for the peptides of 1278.9, 1226.7 and 1307.0 Da than those in the HC group (Figs. 2 and 3A). Three peptides (experimental *m/z* values: 2474.9, 2113.5 and 1226.7 Da) exhibited the greatest distinction when comparing the 2 groups. Hence, we chose these 3 peptides to establish a model by the KNN method with a sensitivity and specificity of 83.33% and 82.86%, respectively, which showed good separation (Fig. 3B).

3.4. Decision tree classification and the ROC curve

As depicted in Fig. 4, subjects were predicted as HC with an intensity ≤ 314 for the peptide at 2474.9 Da, >16 for the peptide at 1226.7 Da and ≤ 230 for the peptide at 2113.5 Da. Subjects were predicted as CKD undergoing haemodialysis with an intensity ≤ 314 for the peptide at 2474.9 Da and ≤ 16 for the peptide at 1226.7 Da, or with an intensity ≤ 314 for the peptide at 2474.9 Da, > 16 for the peptide at 1226.7 Da and > 230 for the peptide at 2113.5 Da or with an intensity > 314 for the peptide at 2474.9 Da. A total of 76.67% of the HD patients were correctly predicted as HD, while 94.29% of HCs were correctly predicted.

From the ROC curve analysis shown in Fig. 5, the area under the curve (AUC) was 0.855 (CI: 0.757–0.954; $p < 0.001$).

3.5. The correlation between candidate salivary peptidomic biomarkers and serum parameters

There were significant positive correlations between the intensities of the peptide at 2113.5 Da and the levels of serum creatinine in the HC group ($p = 0.037$, $r = 0.306$) and in the HD group ($p = 0.041$, $r = 0.322$). In addition, a significant positive correlation was observed between the intensities of the peptide at 2113.5 Da and the levels of blood urea in the HD group ($p = 0.014$), although the correlation was not very strong ($r = 0.402$). No statistically significant correlations were found in the analyses between the other salivary peptidomic biomarkers of interest and serum parameters ($p > 0.05$). The raw data of correlations are shown in Supplementary Table 4.

3.6. Identification of salivary peptides

Based on Q-Exactive mass spectrometry and Mascot, eight differentially expressed peptides were successfully identified. Four peptides (experimental *m/z* values: 2474.9, 2618.5, 2255.4 and 2343.7 Da) were identified as segments of the salivary Histatin-1. Furthermore, the peptides with experimental *m/z* values of 2113.5, 1226.7, 1307.0 and 1278.9 Da were predicted to be segments of Mucin-7, salivary acidic proline-rich phosphoprotein 1/2 (aPRP 1/2), submaxillary gland androgen-regulated protein 3B (SMR3B) and Histatin-3, respectively. Detailed information is shown in Table 1.

4. Discussion

Previous studies that relied on methods including enzymatic assays, 2-dimensional electrophoresis (2-DE), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and MALDI-TOF MS have revealed renal disease-related changes that manifested as elevated urea and creatinine, abnormal proteinuria, and altered electrolytes in serum and urine [9, 22-24]. Despite their widespread use for assessing nephropathy, serum and urine analyses have several drawbacks. In the present study, we used high-throughput techniques to identify salivary peptidomic biomarkers of CKD. We found significant differences in mass spectra peak intensities between the 2 groups, indicating that the salivary proteomics methods will open new research avenues for identifying biomarkers for CKD.

Human saliva is a promising body fluid for assisting in disease diagnosis and prognosis because it can be used to assess the serum levels of metabolic by-products and electrolytes via a simple, safe, quick, inexpensive, and non-invasive collection method [25]. In addition, saliva can be easily stored and its collection is appropriate for all age groups, thus making saliva analyses applicable for screening large populations [26]. Saliva collection can be performed frequently for monitoring disease progression and responses to treatment over time, which may represent a boon to patients suffering from clotting disorders and may decrease anxiety, and discomfort, blood loss, and the potential risk of blood-borne diseases associated with blood collection [13]. The identification of salivary peptides will not only form a foundation for the discovery of potential biomarkers for disease detection but also deepen the understanding of disease pathogenesis.

In this study, we used MALDI-TOF MS techniques combined with WCX magnetic beads

as a sample preparation and proteome fractionation method, which has been recognized as a powerful research tool [27], owing to advantages such as suitability for automation in high-throughput analysis and long-term reproducibility. MALDI-TOF MS, a rapid, high-throughput, inexpensive and reliable technique with high sensitivity and resolution, has been extensively applied to various fields of medicine to analyse peptides, such as dental caries [20], systemic lupus erythaematosus [28], lung adenocarcinoma [29], systemic sclerosis and Sjögren's syndrome [15], and nephrology in particular, as previously mentioned. Benefitting from the properties of strong anionic exchange, hydrophobic/reversed phase, hydrophilic/normal phase and immobilized metal affinity capture [30], WCX magnetic beads separate proteins and/or peptides of different isoelectric points and capture more peptides in the low-molecular-mass range (1–10,000 Da) proteins and low-concentration range [31, 32]. Based on the results of MALDI-TOF MS, we identified some proteins as potential biomarkers by UPLC-MS/MS. High-throughput liquid chromatography (LC) allows the primary separation of peptide mixtures in accordance with the properties of the molecules, followed by the second mass spectrometry (MS) dimension to obtain amino acid sequences, expanding the range of molecular weights for the separated substances. Therefore, UPLC-MS/MS shows absolute advantages in improving the analysis efficiency and throughput with excellent selectivity and sensitivity [33]. Research by Navarro-Muñoz et al. revealed differentially expressed urinary peptides that can distinguish between glomerular kidney disease patients and healthy subjects using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [34]. In addition, Ngo et al. reported a nano-LC-MS/MS platform with high sensitivity for the identification of proteins in

human gingival crevicular fluid [35]. With respect to saliva analysis for CKD, Suzuki et al. found that evaluating saliva samples by UPLC-MS/MS was useful for determining creatinine-related molecules and evaluating haemodialysis in CKD patients [36]. The integration of such technologies provides us with novel high-efficiency methods to discover candidate salivary biomarkers that could differentiate CKD patients from healthy subjects.

We found that the salivary pH values in the HD group were significantly higher than those in the HC group ($p < 0.01$), which was similar to the results of previous studies. Many studies have verified that salivary levels of urea are significantly elevated in CKD patients [13]. Salivary urea is metabolized by oral microflora to form ammonia and carbon dioxide, which raises the salivary pH [37] and may provide a protective effect on enamel demineralization.

We chose 3 peptides (experimental *m/z* values: 2474.9, 2113.5 and 1226.7 Da) to establish a discrimination model by the KNN method (Fig. 3B). As shown in the figure, the plots in the disease group were more diffuse, while the plots were more concentrated in the control group. We presumed that this result may be partly explained by the coaction of various confounding factors on haemodialysis, and furthermore, the results also provide initial evidence for differential peptide expression between the 2 groups.

The results of the decision tree (Fig. 4) suggested that the 3 peptides (experimental *m/z* values: 2474.9, 2113.5 and 1226.7 Da) in the KNN model were useful in differentiating HD patients from HCs. Concomitantly, the candidate clinical predictor thresholds for CKD on the intensities of the salivary peptides were provided. Furthermore, the ROC curve analysis revealed an AUC of 0.855 (Fig. 5), indicating excellent diagnostic efficacy of the KNN

model.

Creatinine is a breakdown product of creatine phosphate in muscle, and blood urea is a major nitrogenous end product of protein and amino acid catabolism. These 2 small molecule metabolites are primarily excreted by kidneys; moreover, blocked excretion caused by impaired renal function would result in an increase in blood levels of creatinine as well as urea. Hence, serum creatinine and blood urea are good indicators to assess renal functions [38]. We attempted to find the association between the effective blood indicators and the 3 peptides (experimental *m/z* values: 2474.9, 2113.5 and 1226.7 Da), which were chosen to establish the discrimination model. Although the correlation was weak, the significant positive correlation between the peptide at 2113.5 Da and serum creatinine/blood urea levels may contribute to the evidence of salivary peptides as candidate biomarkers of CKD.

Histatin-1, which is a common isoform of the Histatin family of peptides that are secreted by parotid and submandibular glands, exhibited higher expression in the HD group (Table 1). The functions of Histatin include buffering, wound healing, anti-candida, antiviral, and antibacterial actions, and balancing mineral formation [39]. Histatin-1 can be selectively absorbed by hydroxyapatite crystals, participating in the formation of acquired enamel pellicle and simultaneously protecting against proteolytic degradation and demineralization [40]. Moreover, it was verified that Histatin-1 indirectly induces wound healing by stimulating epithelial migration [41]. The presence of Histatin-1 is indispensable for maintaining the stability of the saliva environment. Trindade et al. reported that Histatin-1 may play an important role in the defence response against oral pathogens as a mediator in the healing process of infected periodontal areas [42]. Borawski et al. found that CKD

patients under haemodialysis displayed more periodontal lesions than controls did [43]. In our study, although we hope to ascertain the subjects' periodontal status, unfortunately, the majority of CKD HD patients could not tolerate complete systematic periodontal examination due to compromised physical and mental health and poor compliance. Notwithstanding, an experienced specialist performed the oral examinations and subjects with symptoms of moderate and severe periodontitis (marked gingival inflammation, periodontal abscess, teeth mobility and migration, root exposure, etc.) were excluded as far as possible. Furthermore, CKD patients and HCs with no significant differences in periodontal inflammatory status were enrolled on the basis of the inclusion/exclusion criteria in the study. That might reduce the influence of periodontitis on our findings to some extent, nevertheless, it also reminds us to be cautious of extrapolating the conclusions of our study. In consideration of the complicated physiological and pathological circumstances of CKD patients undergoing haemodialysis, we speculated that the significant increase in Histatin-1 in the HD group seems to be a counterbalance of the destructive reactions held by inflammatory factors in view of its antimicrobial and wound healing properties. However, there are few studies on the role of Histatin-3 in CKD.

Previous studies have identified that Mucin-7, a type of mucin secreted in the oral cavity, can entrap and agglutinate bacteria, fungi and viral particles with high affinity for microorganisms [44]. In this study, the expression level of Mucin-7 was higher in the HD group (Table 1). As a major component of the salivary pellicle that coats the tooth, Mucin-7 also helps regulate bacterial adhesion in conjunction with acidic proline-rich proteins [45]. Additionally, Mucin-7 can protect other proteins from proteolysis, modulate the biological

activity of proteins and serve as a delivery system for the distribution of salivary proteins throughout the oral cavity [46]. Alterations in mucin expression or glycosylation accompany the development of cancer and influence cellular growth, discrimination, transformation, adhesion, invasion and immune surveillance [47]. Nguyen Hoang et al. [48] showed that high Mucin-7 expression was an independent predictor of adverse clinical outcomes in patients with clear-cell renal cell carcinoma. The expression of Mucin-7 may be a potential urinary marker for carcinoma in situ and invasive bladder cancer with high sensitivity and specificity [49]. These studies confirmed the role of Mucin-7 in urinary system carcinomas, by which Mucin-7 was predicted to interfere with the inflammatory process and immune response. We hypothesize that increased Mucin-7 might be released into the circulation due to the inflammatory damage in CKD, implying the possibility that Mucin-7 may serve as a potential salivary disease biomarker.

Salivary acidic proline-rich phosphoproteins (aPRPs) exert calcium hydroxide-binding properties and therefore participate in the formation of acquired pellicle on the surface of teeth [50]. Vitorino et al. investigated the negative correlation between the level of aPRP 1/2 and the number of decayed, missing, and filled teeth, indicating its protective role [51]. In addition, Thorman et al. reported that haemodialysis patients had worse DMFT index scores than HCs did [52]. The recommended low-protein diet with a frequent and high intake of carbohydrates for pre-dialysis CKD patients likely increased the risk of developing caries [53]. Alterations in salivary constituents, decreased flow rate and the accompanying xerostomia are relatively common in patients undergoing haemodialysis [37], which also promotes dental caries. Salivary aPRP, as an inhibitor of calcium phosphate crystal growth

from saliva, may provide a protective, reparative, but stable environment for dental enamel [54]. We speculated that the decreased expression observed in the HD group might be associated with the oral health status of haemodialysis patients.

SMR3B was identified as a Porphyromonas gingivalis lipopolysaccharide-binding protein (LBP) in human saliva, and LBPs are known to be involved in bacterial adhesion and colonization, antimicrobial functions and the modulation of immune responses [55]. Ren et al. reported that LBP was significantly elevated in the healthy population compared with periodontitis patients [56]. Consequently, the reduction in SMR3B may be linked with the inferior periodontal condition of CKD patients to a certain extent.

Some limitations of the present study must be taken into account. It is difficult to identify all the different sources of peptide peaks in the saliva, as some of the peptides may originate from some microorganisms or other sources. Additionally, the protein databases are far from complete, which contributes to difficulty in peptide identification. Although we tried to match the 2 groups during the recruitment process as far as possible based on our research purpose, the intricate pathophysiological states combined with the interaction of various confounding factors in HD patients, including periodontitis as previously mentioned, may still affect the outcomes to some extent. Our cross-sectional analytical study partially revealed the differential expression of salivary proteins at the current stage of disease development. In our subsequent work, a larger series of patients and longitudinal studies are necessary to validate the roles of the identified proteins in CKD with a minimum bias. Likewise, more efforts should be dedicated to investigating the function and structures of these candidate biomarkers to deepen the understanding of the intrinsic mechanisms of CKD at the molecular level.

5. Conclusions

The current findings provide initial evidence that proteomics methods can facilitate the identification of salivary biomarkers for CKD. Histatin-1, Mucin-7 and aPRP 1/2 are expected to be candidate salivary biomarkers of CKD for patients undergoing haemodialysis, which may lay a foundation for their potential utilization in disease detection, diagnosis and therapeutic monitoring. Further improvements in technologies will drive research on salivary disease biomarkers and saliva, as a promising non-invasive diagnostic biofluid, might provide great value to researches of chronic systemic diseases.

The following are the supplementary data related to this article.

Supplementary Table 1. Demographic information and salivary pH values of subjects in the 2 groups.

Supplementary Table 2. Comparison of the 199 peptide peaks detected simultaneously in the 2 groups.

Supplementary Table 3. Significance, average intensities and frequencies of the eight peptides differentially expressed between the 2 groups.

Supplementary Table 4. The intensities of the 3 peptides used to establish the discrimination model and levels of serum parameters.

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References

[1] L.A. Inker, B.C. Astor, C.H. Fox, T. Isakova, J.P. Lash, C.A. Peralta, M. Kurella Tamura, H.I. Feldman, KDOQI US commentary on the 2012 KDIGO clinical practice guideline for the evaluation and management of CKD, Am. J. Kidney Dis. 63(5) (2014) 713-735.

[2] Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, KDIGO 2012 Clinical practice guideline for the evaluation and management of chronic kidney disease, Kidney Int. Suppl. (2013) 1-150.

[3] A.C. Webster, E.V. Nagler, R.L. Morton, P. Masson, Chronic Kidney Disease, The Lancet 389(10075) (2017) 1238-1252.

[4] L. Zhang, F. Wang, L. Wang, W. Wang, B. Liu, J. Liu, M. Chen, Q. He, Y. Liao, X. Yu, N. Chen, J.-e. Zhang, Z. Hu, F. Liu, D. Hong, L. Ma, H. Liu, X. Zhou, J. Chen, L. Pan, W. Chen, W. Wang, X. Li, H. Wang, Prevalence of chronic kidney disease in China: a cross-sectional survey, The Lancet 379(9818) (2012) 815-822.

[5] A.S. Levey, R. Atkins, J. Coresh, E.P. Cohen, A.J. Collins, K.U. Eckardt, M.E. Nahas, B.L. Jaber, M. Jadoul, A. Levin, N.R. Powe, J. Rossert, D.C. Wheeler, N. Lameire, G. Eknoyan, Chronic kidney disease as a global public health problem: approaches and initiatives - a position statement from Kidney Disease Improving Global Outcomes, Kidney Int. 72(3) (2007) 247-59.

[6] GBD 2013 Mortality and Causes of Death Collaborators, Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013, The Lancet 385(9963) (2015) 117-171.

[7] R.A. Nugent, S.F. Fathima, A.B. Feigl, D. Chyung, The burden of chronic kidney disease on developing nations: a 21st century challenge in global health, Nephron. Clin. Pract. 118(3) (2011) c269-277.

[8] A.S. Levey, C. Becker, L.A. Inker, Glomerular filtration rate and albuminuria for detection and staging of acute and chronic kidney disease in adults: a systematic review, JAMA 313(8) (2015) 837-846.

[9] R. Renda, Can salivary creatinine and urea levels be used to diagnose chronic kidney disease in children as accurately as serum creatinine and urea levels? A case-control study, Ren. Fail. 39(1) (2017) 452-457.

[10] M. Castagnola, P.M. Picciotti, I. Messana, C. Fanali, A. Fiorita, T. Cabras, et al., Potential applications of human saliva as diagnostic fluid, Acta Otorhinolaryngol Ital. 31(6) (2011) 347-357.

[11] G. Bayraktar, R. Kazancioglu, S. Bozfakioglu, A. Yildiz, E. Ark, Evaluation of salivary parameters and dental status in adult hemodialysis patients, Clin. Nephrol. 62(5) (2004) 380-383.

[12] I. Tomas, J.S. Marinho, J. Limeres, M.J. Santos, L. Araujo, P. Diz, Changes in salivary composition in patients with renal failure, Arch. Oral Biol. 53(6) (2008) 528-532.

[13] T.J. Lasisi, Y.R. Raji, B.L. Salako, Salivary creatinine and urea analysis in patients with chronic kidney disease: a case control study, BMC Nephrol. 17 (2016) 10.

[14] N. Rosa, M.J. Correia, J.P. Arrais, P. Lopes, J. Melo, J.L. Oliveira, M. Barros, From the salivary proteome to the OralOme: comprehensive molecular oral biology, Arch. Oral Biol. 57(7) (2012) 853-864.

[15] S.K. Al-Tarawneh, M.B. Border, C.F. Dibble, S. Bencharit, Defining salivary biomarkers using mass spectrometry-based proteomics: a systematic review, OMICS 15(6) (2011) 353-361.

[16] F. Graterol, M. Navarro-Muñoz, M. Ibernon, D. López, M.I. Troya, V. Pérez, et al., Poor histological lesions in IgA nephropathy may be reflected in blood and urine peptide profiling, BMC Nephrol. (14) (2013) 82.

[17] J. Yang, J. Yang, Y. Gao, L. Zhao, L. Liu, Y. Qin, X. Wang, T. Song, C. Huang, Identification of potential serum proteomic biomarkers for clear cell renal cell carcinoma, PLoS One 9(11) (2014) e111364.

[18] A. Aggarwal, R. Gupta, V.S. Negi, L. Rajasekhar, R. Misra, P. Singh, V. Chaturvedi, S. Sinha, Urinary haptoglobin, alpha-1 anti-chymotrypsin and retinol binding protein identified by proteomics as potential biomarkers for lupus nephritis, Clin. Exp. Immunol. 188(2) (2017) 254-262.

[19] A. Carlen, H. Hassan, P. Lingstrom, The 'strip method': a simple method for plaque pH assessment, Caries Res. 44(4) (2010) 341-344.

[20] X. Sun, X. Huang, X. Tan, Y. Si, X. Wang, F. Chen, S. Zheng, Salivary peptidome profiling for diagnosis of severe early childhood caries, J. Transl. Med. 14(1) (2016) 240.

[21] S. Ao, X. Sun, X. Shi, X. Huang, F. Chen, S. Zheng, Longitudinal investigation of salivary proteomic profiles in the development of early childhood caries, J. Dent. 61 (2017) 21-27.

[22] J. Wu, N. Wang, J. Wang, Y. Xie, Y. Li, T. Liang, J. Wang, Z. Yin, K. He, X. Chen, Identification of a uromodulin fragment for diagnosis of IgA nephropathy, Rapid

Commun. Mass Spectrom 24(14) (2010) 1971-1978.

[23] S. Minami, K. Matsumoto, R. Nagashio, D. Hagiuda, E. Fukuda, N. Goshima, M. Hattori, B. Tsuchiya, K. Hachimura, S.X. Jiang, M. Saegusa, M. Iwamura, Y. Sato, Analysis of Autoantibodies Related to Tumor Progression in Sera from Patients with High-grade Non-muscle-invasive Bladder Cancer, Anticancer Res. 37(12) (2017) 6705-6714.

[24] V.P. Rodrigues, M.M. Franco, C.P. Marques, R.C. de Carvalho, S.A. Leite, A.L. Pereira, B.B. Benatti, Salivary levels of calcium, phosphorus, potassium, albumin and correlation with serum biomarkers in hemodialysis patients, Arch. Oral Biol. 62 (2016) 58-63.

[25] K.E. Kaczor-Urbanowicz, C. Martin Carreras-Presas, K. Aro, M. Tu, F. Garcia-Godoy, D.T. Wong, Saliva diagnostics - Current views and directions, Exp. Biol. Med. (Maywood) 242(5) (2017) 459-472.

[26] T. Pfaffe, J. Cooper-White, P. Beyerlein, K. Kostner, C. Punyadeera, Diagnostic potential of saliva: current state and future applications, Clin. Chem. 57(5) (2011) 675-687.

[27] J. Hajduk, J. Matysiak, Z.J. Kokot, Challenges in biomarker discovery with MALDI-TOF MS, Clin. Chim. Acta 458 (2016) 84-98.

[28] Y. Dai, C. Hu, L. Wang, Y. Huang, L. Zhang, X. Xiao, Y. Tan, Serum peptidome patterns of human systemic lupus erythematosus based on magnetic bead separation and MALDI-TOF mass spectrometry analysis, Scand. J. Rheumatol. 39(3) (2010) 240-246.

[29] X.L. Lin, S.Y. Yang, J. Du, Y.X. Tian, L.N. Bu, S.F. Huo, et al., Detection of lung adenocarcinoma using magnetic beads based matrix-assisted laserdesorption/ionization time-of-flight mass spectrometry serum protein profiling, Chin. Med. J. 123 (2010)

34-39.

[30] Q. He, L. Shao, J. Yu, S. Ji, H. Wang, Y. Mao, et al., Urinary Proteome Analysis by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry with Magnetic Beads for Identifying the Pathologic Presentation of Clinical Early IgA Nephropathy, J. Biomed. Nanotechnol. 8(1) (2012) 133-139.

[31] S. Baumann, U. Ceglarek, G.M. Fiedler, J. Lembcke, A. Leichtle, J. Thiery, Standardized approach to proteome profiling of human serum based on magnetic bead separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Clin. Chem. 51(6) (2005) 973-980.

[32] O. Deutsch, Y. Fleissig, B. Zaks, G. Krief, D. Aframian, A. Palmon, An approach to remove alpha amylase for proteomic analysis of low abundance biomarkers in human saliva, Electrophoresis 29(20) (2008) 4150-4157.

[33] H. Tsutsui, T. Mochizuki, T. Maeda, I. Noge, Y. Kitagawa, J.Z. Min, et al., Simultaneous determination of DL-lactic acid and DL-3-hydroxybutyric acid enantiomers in saliva of diabetes mellitus patients by high-throughput LC-ESI-MS/MS, Anal. Bioanal. Chem. 404(6-7) (2012) 1925-1934.

[34] M. Navarro-Munoz, M. Ibernon, J. Bonet, V. Perez, M.C. Pastor, B. Bayes, et al., Uromodulin and alpha(1)-antitrypsin urinary peptide analysis to differentiate glomerular kidney diseases, Kidney Blood Press. Res. 35(5) (2012) 314-325.

[35] L.H. Ngo, P.D. Veith, Y.Y. Chen, D. Chen, I.B. Darby, E.C. Reynolds, Mass spectrometric analyses of peptides and proteins in human gingival crevicular fluid, J. Proteome Res. 9(4) (2010) 1683-1693.

[36] M. Suzuki, M. Furuhashi, S. Sesoko, K. Kosuge, T. Maeda, K. Todoroki, et al., Determination of creatinine-related molecules in saliva by reversed-phase liquid chromatography with tandem mass spectrometry and the evaluation of hemodialysis in chronic kidney disease patients, Anal. Chim. Acta 911 (2016) 92-99.

[37] M. Honarmand, L. Farhad-Mollashahi, A. Nakhaee, F. Sargolzaie, Oral manifestation and salivary changes in renal patients undergoing hemodialysis, J. Clin. Exp. Dent. 9(2) (2017) e207-210.

[38] D. Pandya, A.K. Nagrajappa, K.S. Ravi, Assessment and Correlation of Urea and Creatinine Levels in Saliva and Serum of Patients with Chronic Kidney Disease, Diabetes and Hypertension- A Research Study, J. Clin. Diagn. Res. 10(10) (2016) ZC58-ZC62.

[39] Z. Khurshid, S. Najeeb, M. Mali, S. Moin, S.Q. Raza, S. Zohaib, F. Sefat, M.S. Zafar, Histatin peptides: Pharmacological functions and their applications in dentistry, Saudi. Pharm. J. 25(1) (2017) 25-31.

[40] E.E. McDonald, H.A. Goldberg, N. Tabbara, F.M. Mendes, W.L. Siqueira, Histatin-1 resists proteolytic degradation when adsorbed to hydroxyapatite, J. Dent. Res. 90 (2011) 268–272.

[41] M.J. Oudhoff, J.G. Bolscher, K. Nazmi, H. Kalay, W. van 't Hof, A.V. Amerongen, E.C. Veerman, Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay, FASEB J. 22 (2008) 3805–3812.

[42] F. Trindade, F. Amado, R.P. Oliveira-Silva, A.L. Daniel-da-Silva, R. Ferreira, J. Klein, R. Faria-Almeida, P.S. Gomes, R. Vitorino, Toward the definition of a peptidome signature

and protease profile in chronic periodontitis, Proteomics Clin. Appl. 9(9-10) (2015) 917-927.

[43] J. Borawski, M. Wilczynska-Borawska, W. Stokowska, M. Mysliwiec, The periodontal status of pre-dialysis chronic kidney disease and maintenance dialysis patients, Nephrol. Dial. Transplant. 22(2) (2007) 457-464.

[44] S. Gorr, Antimicrobial peptides of the oral cavity, Periodontology 2000 51 (2009) 152–180.

[45] U. Lendenmann, J. Grogan, F.G. Oppenheim, Saliva and dental pellicle--a review, Adv. Dent. Res. 14 (2000) 22-28.

[46] L.S. Bruno, X. Li, L. Wang, R.V. Soares, C.C. Siqueira, F.G. Oppenheim, R.F. Troxler, G.D. Offner, Two-hybrid analysis of human salivary mucin MUC7 interactions, Biochim. Biophys. Acta 1746(1) (2005) 65-72.

[47] M.A. Hollingsworth, B.J. Swanson, Mucins in cancer: protection and control of the cell surface, Nat. Rev. Cancer 4(1) (2004) 45-60.

[48] S. Nguyen Hoang, Y. Liu, L. Xu, Y. Chang, L. Zhou, Z. Liu, Z. Lin, J. Xu, High mucin-7 expression is an independent predictor of adverse clinical outcomes in patients with clear-cell renal cell carcinoma, Tumour Biol. 37(11) (2016) 15193-15201.

[49] M. Retz, J. Lehmann, E. Amann, B. Wullich, C. Roder, M. Stockle, Mucin 7 and cytokeratin 20 as new diagnostic urinary markers for bladder tumor, J. Urol. 169(1) (2003) 86-89.

[50] T.K. Fabian, P. Hermann, A. Beck, P. Fejerdy, G. Fabian, Salivary defense proteins: their network and role in innate and acquired oral immunity, Int. J. Mol. Sci. 13(4) (2012)

4295-4320.

[51] R. Vitorino, S. de Morais Guedes, R. Ferreira, M.J. Lobo, J. Duarte, A.J. Ferrer-Correia, et al., Two-dimensional electrophoresis study of in vitro pellicle formation and dental caries susceptibility, Eur. J. Oral Sci. 114 (2006) 147-153.

[52] R. Thorman, M. Neovius, B. Hylander, Clinical findings in oral health during progression of chronic kidney disease to end-stage renal disease in a Swedish population, Scand. J. Urol. Nephrol. 43(2) (2009) 154-159.

[53] A. Sheiham, Dietary effects on dental diseases, Public Health Nutr. 4(2B) (2001) 569-591.

[54] D.H. Schlesinger, D.I. Hay, Complete covalent structure of a proline-rich phosphoprotein, PRP-2, an inhibitor of calcium phosphate crystal growth from human parotid saliva, Int. J. Pept. Protein Res. 27(4) (1986) 373-379.

[55] S. Choi, J.E. Baik, J.H. Jeon, K. Cho, D.G. Seo, K.Y. Kum, C.H. Yun, S.H. Han, Identification of Porphyromonas gingivalis lipopolysaccharide-binding proteins in human saliva, Mol. Immunol. 48(15-16) (2011) 2207-2213.

[56] L. Ren, L. Jin, W.K. Leung, Local expression of lipopolysaccharide-binding protein in human gingival tissues, J. Periodontal Res. 39(4) (2004) 242-248.

Table 1. Peptide sequences identified by Q-Exactive MS among those differentially expressed by MALDI-TOF MS.

Experimental *m/z* value	**Peptide sequence**	**Proteins**	**Tendency** [a]	**Theoretical *m/z* value**
2474.9	H.HSHREFPFYGDYGSNYLYDN.-	Histatin-1	↑	2480.0491
2255.4	S.HREFPFYGDYGSNYLYDN.-	Histatin-1	↑	2255.9546
2343.7	H.SHREFPFYGDYGSNYLYDN.-	Histatin-1	↑	2342.9860
2618.5	K.HHSHREFPFYGDYGSNYLYDN.-	Histatin-1	↑	2617.1065
2113.5	K.SHFELPHYPGLLAHQKPF.I	Mucin-7	↑	2117.0885
1226.7	Q.GPPQQGGHPRPP.R	aPRP 1/2	↓	1223.6164
1307.0	Y.GPGRIPPPPPAPY.G	SMR3B	↓	1314.7099
1278.9	F.HEKHHSHRGY.R	Histatin-3	↓	1286.6018

m/z mass-to-charge ratio

[a] Tendency refers to the trend in the intensity of *m/z* values between the two groups. ↑, Higher intensity in the HD group than in the HC group; ↓, lower intensity in the HD group than in the HC group.

Fig. 1. Complete mass spectra of the peptide samples in the range of 1,000–10,000 Da. These data were from representative healthy individuals (A) and patients with CKD undergoing haemodialysis (B). *m/z*, mass-to-charge ratio.

Fig. 2. Three-dimensional *m/z* ratio-intensity maps. The maps show significantly different intensities for the 5 representative peptides at 1226.7, 2474.9, 2113.5, 1307.0 and 1278.9 Da. Green, HC group; red, HD group.

Fig. 3. Histograms and scatter plots of the two groups. (A) Histogram of the mass spectra intensities from the 2 groups show increased expression of the peptides at 2474.9, 2618.5, 2255.4, 2343.7 and 2113.5 Da and decreased expression of the peptides at 1278.9, 1226.7 and 1307.0 Da in the HD group. **$p < 0.01$; ***$p < 0.001$. (B) Scatter plots of the two groups established by combining the peptides at 2474.9, 2113.5 and 1226.7 Da.

Fig. 4. Decision tree analysis. The peptides and the intensity thresholds for classification as nodes are shown in the ovals. Class = HD/HC, representing predicted-HD/HC as terminal nodes, are shown in the rectangles which contained the real numbers of HD/HC.

Fig. 5. Receiver operating characteristic (ROC) curve. The ROC curve analysis for the peptides at 2113.5, 1226.7 and 2474.9 Da to distinguish HD patients from HCs revealed an area under the curve (AUC) of 0.855 (CI: 0.757–0.954; $p < 0.001$).

Highlights:

- Salivary proteomics will open new avenues of research on biomarkers for CKD.
- The mass spectra peak intensities differed significantly between the two groups.
- The CKD patients undergoing haemodialysis showed higher salivary pH.
- The salivary peptidomic biomarkers correlated significantly with serum parameters.
- Histatin-1, Mucin-7 and aPRP 1/2 may be candidate salivary biomarkers for CKD.

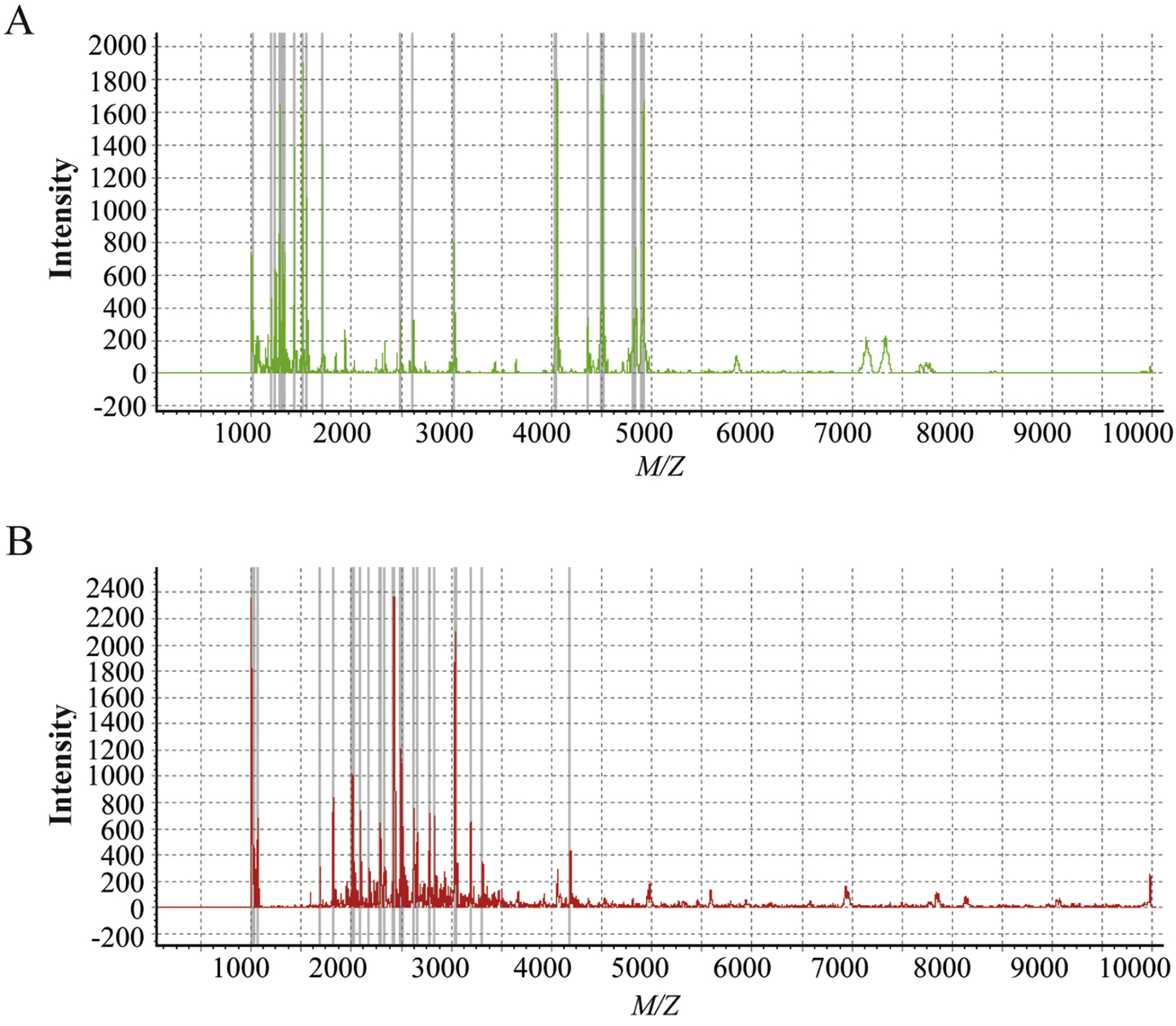
A
Intensity
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
M/Z
B
Intensity
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
M/Z

Figure 1

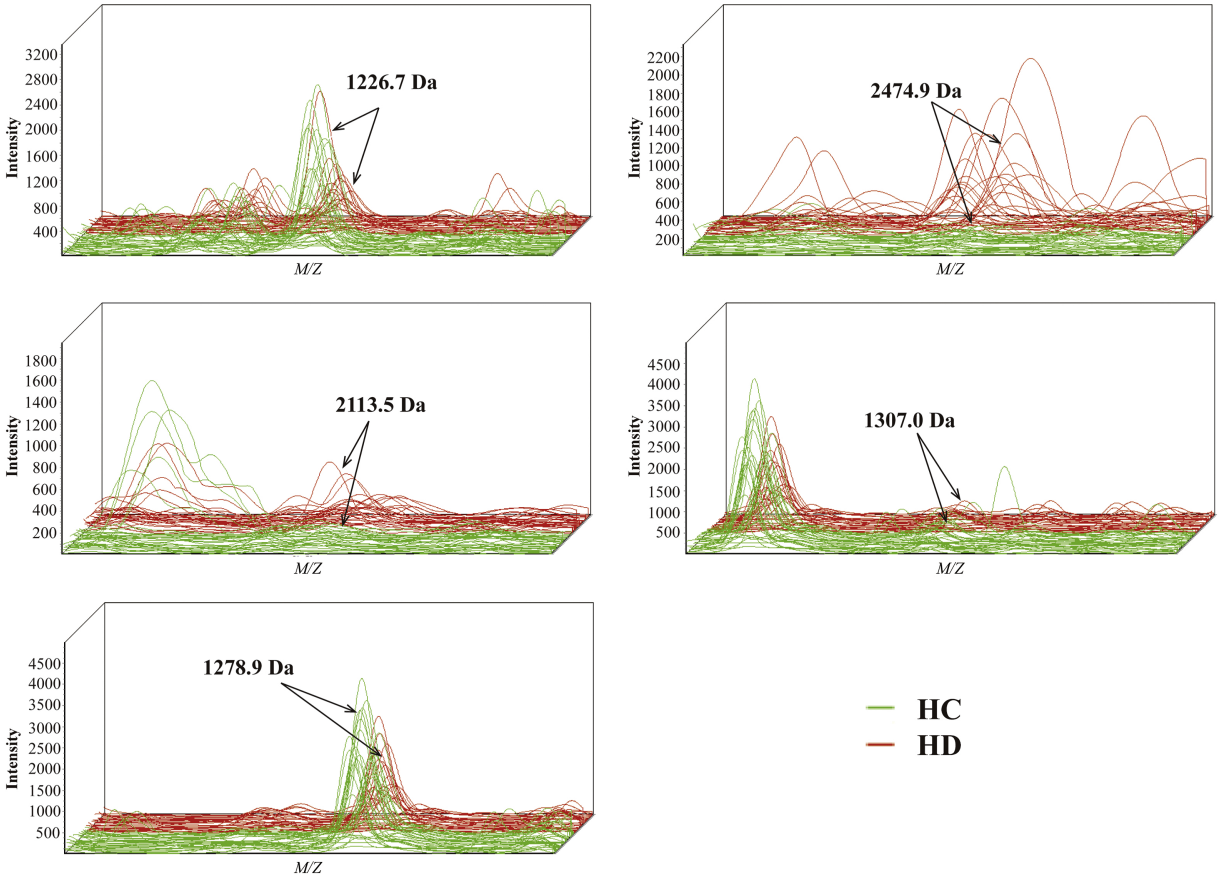
1226.7 Da
2474.9 Da
2113.5 Da
1307.0 Da
1278.9 Da
Intensity
M/Z
HC
HD

Figure 2

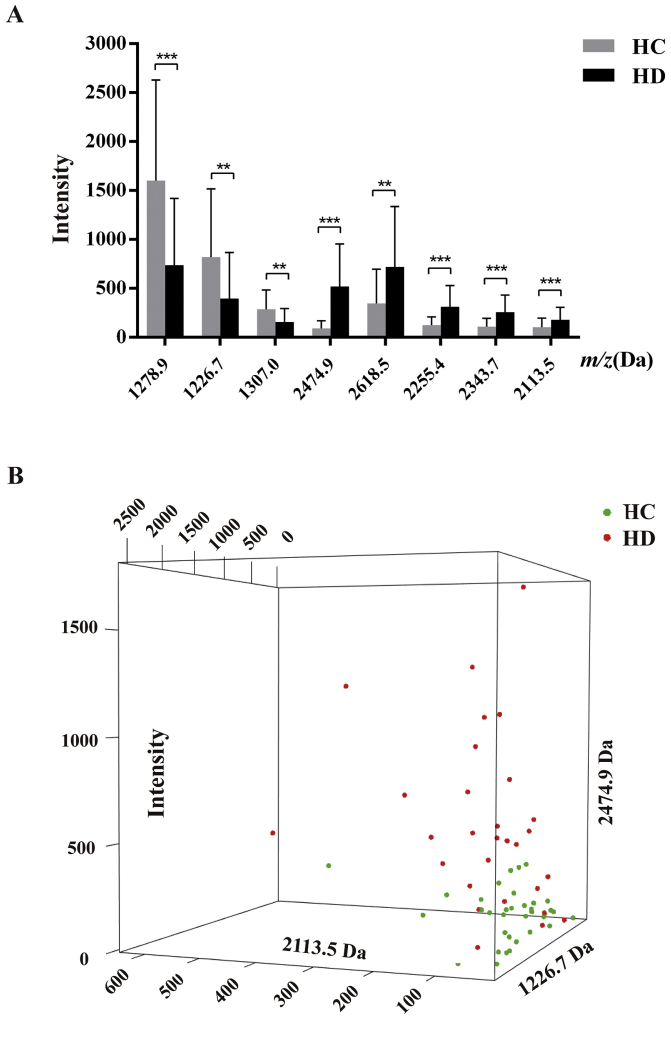
A
HC
HD
Intensity
3000
2500
2000
1500
1000
500
0
***
**
**
***
**
***
***
***
1278.9
1226.7
1307.0
2474.9
2618.5
2255.4
2343.7
2113.5
m/z(Da)
B
HC
HD
2500
2000
1500
1000
500
0
1500
1000
500
0
Intensity
2474.9 Da
2113.5 Da
1226.7 Da
600
500
400
300
200
100

Figure 3

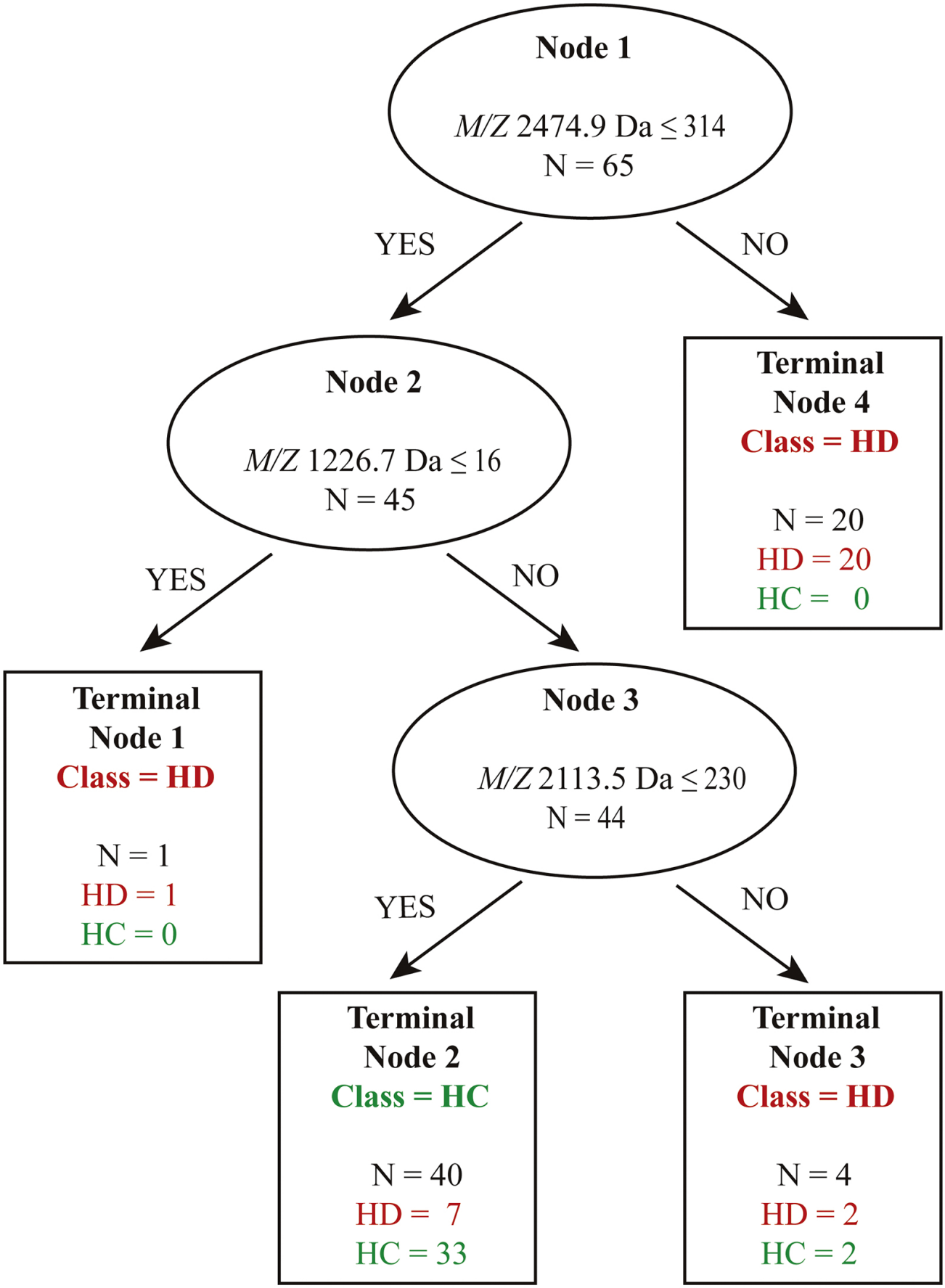
Node 1
M/Z 2474.9 Da ≤ 314
N = 65
YES
NO
Node 2
M/Z 1226.7 Da ≤ 16
N = 45
Terminal Node 4
Class = HD
N = 20
HD = 20
HC = 0
YES
NO
Terminal Node 1
Class = HD
N = 1
HD = 1
HC = 0
Node 3
M/Z 2113.5 Da ≤ 230
N = 44
YES
NO
Terminal Node 2
Class = HC
N = 40
HD = 7
HC = 33
Terminal Node 3
Class = HD
N = 4
HD = 2
HC = 2

Figure 4

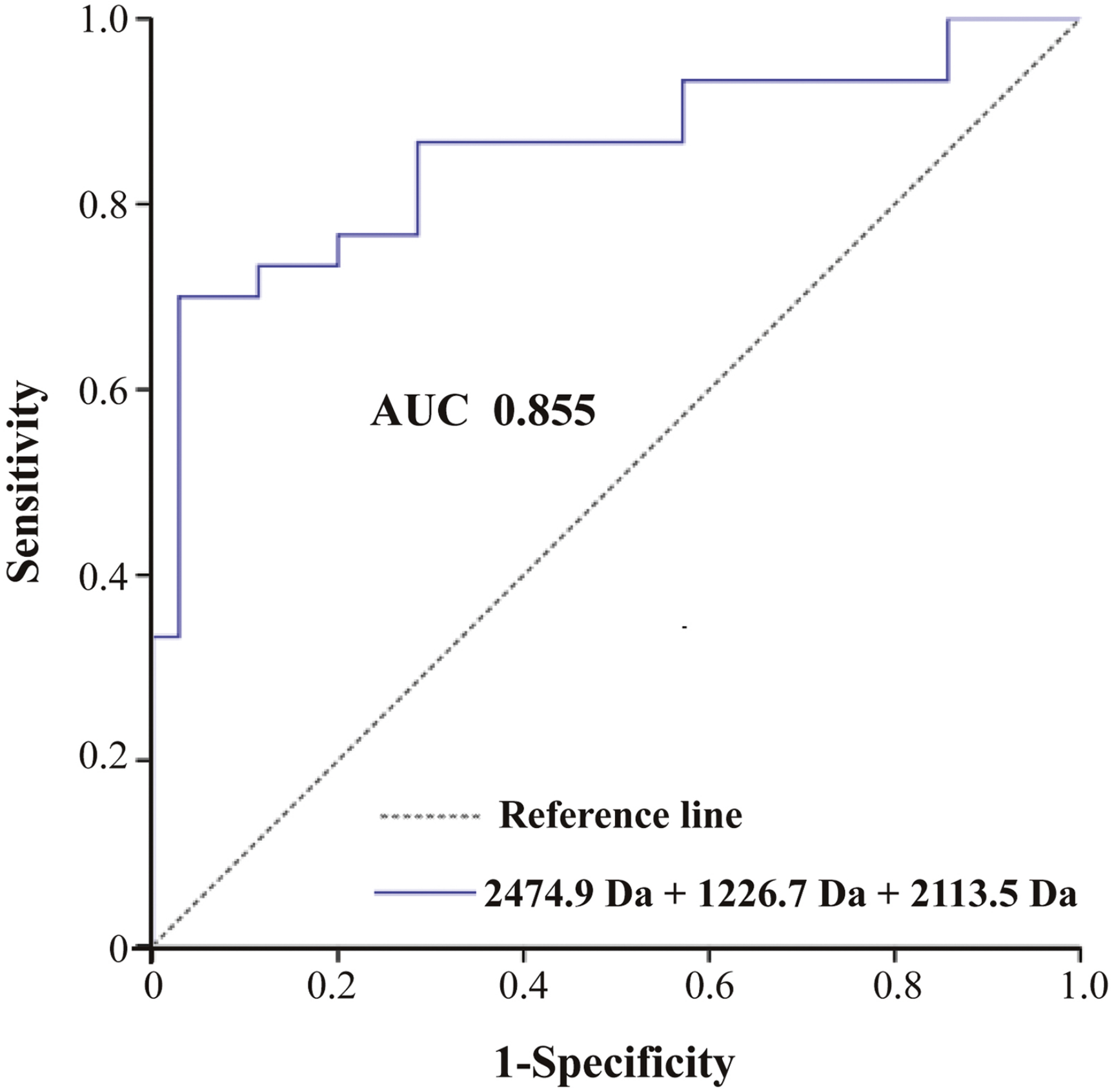
1.0
0.8
0.6
0.4
0.2
0
Sensitivity
AUC 0.855
Reference line
2474.9 Da + 1226.7 Da + 2113.5 Da
0
0.2
0.4
0.6
0.8
1.0
1-Specificity

Figure 5