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Metabolomics Panel Associated with Cystic Fibrosis-Related Diabetes toward Biomarker Discovery

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Cite This: *ACS Omega* 2024, 9, 32873−32880

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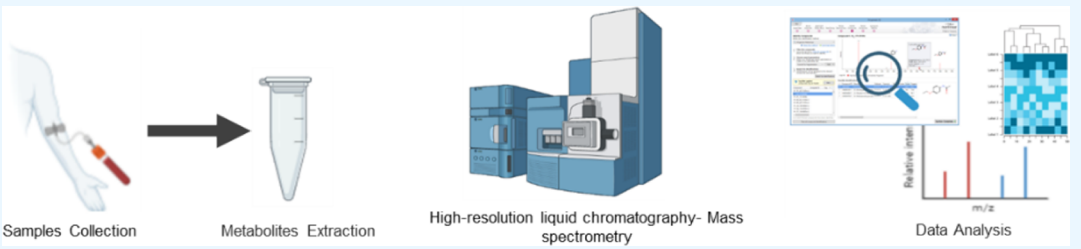


ABSTRACT: The most prevalent comorbidity among cystic fibrosis (CF) patients is cystic fibrosis-related diabetes (CFRD). CFRD has been linked to one of the worse clinical outcomes and a higher mortality. Improved clinical results have been related to earlier diagnosis and treatment of CFRD. Therefore, the present study aimed to investigate the metabolome of human serum of patients with CFRD. This might aid in identifying novel biomarkers linked with the pathophysiology of CFRD and its diagnosis. The liquid chromatography−high-resolution mass spectrometry (LC−HRMS) metabolomics approach was utilized for serum samples from patients with CF (n = 36) and healthy controls (n = 36). Nine patients in the CF group had CFRD, and 27 were non-CFRD patients (nCFRD). A total of 2328 metabolites were significantly altered in CF compared with the healthy control. Among those, 799 significantly dysregulated metabolites were identified between CFRD and nCFRD. Arachidonic acid (AA), ascorbate, and aldarate metabolism were the most common metabolic pathways dysregulated in CF. L-Homocysteic acid (L-HCA) levels were significantly reduced in CF and CFRD compared to the control and nCFRD, respectively. In addition, gamma-glutamylglycine and L-5-hydroxytryptophan (5-HTP) had the highest discrimination between CFRD and nCFRD with AUC (0.716 and 0.683, respectively). These biomarkers might serve as diagnostic biomarkers and aid in understanding potential metabolic changes linked to CF and CFRD.

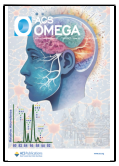
INTRODUCTION

Alterations in a specific gene responsible for encoding the cystic fibrosis transmembrane conductance regulator (CFTR) led to the development of cystic fibrosis (CF). This complex disorder impacts various systems within the body. This condition affects various organs, encompassing the respiratory system, gastrointestinal tract, exocrine pancreas, and hepatobiliary system.[1] The CFTR gene is located on chromosome 7 (7q31.2) and has approximately 2,100 known variants.[2] So far, about 400 CFTR gene mutations have been shown to cause CF.[3] The deletion of a phenylalanine residue at position 508 (F508) is the most common mutation.

The most common nonpulmonary complication of CF is cystic fibrosis-related diabetes (CFRD), which affects approximately half of all individuals with CF at some time.[4] Patients with severe CFTR pathogenic variants (which represent 85% of the patients with CF) that cause pancreatic insufficiency (PI) are more likely to develop CFRD.[5] A lack of insulin due to dysfunctional beta cells is the primary cause of the disease. However, insulin resistance, insufficient functioning of other pancreatic hormones, disruption in the gut-insulin connection, and disruption in the process of insulin clearance are also contributors.[6]

Despite sharing characteristics with types 1 (T1DM) and 2 of diabetes mellitus (T2DM), such as insulin reduction and resistance, CFRD is a distinct type of disease. Unlike T1DM, CF β-cell destruction is not autoimmune and remains insulin-secreting.[7] Instead, aberrant chloride channel activity causes thick viscous secretions that damage the exocrine pancreas.[8] Some of CFRD's distinguishing features include an increased energy expenditure necessitating a very high caloric intake to maintain weight and nutritional status, a deficiency but not absence of insulin secretion due to pancreatic islet deficiency

Received: April 15, 2024
Revised: July 8, 2024
Accepted: July 10, 2024
Published: July 17, 2024



or malfunction, chronic baseline inflammation that periodically flares during infection, and increased energy expenditure.[9]

CFRD is linked to increased morbidity due to a worsening lung and nutritional status and increased risk of microvascular complications, which often start before a diagnosis of CFRD, and increased mortality if CFRD is not treated.[10] If correctly treated, then CFRD diagnosis at an earlier stage may lead to better clinical outcomes and lower mortality rates.

The classic symptoms of diabetes, such as polyuria and polydipsia, may not be present in CFRD, and other symptoms, such as poor growth and an unexpected decline in pulmonary function, are also nonspecific.[11] Moreover, because HbA1c (%) may be normal in the early stages and cases of intermittent hyperglycemia, it should not be used solely as a screening test for CFRD. HbA1c values may be lower than normal because erythrocyte half-life is shortened in CF due to the chronic inflammatory state.[11]

Current guidelines conducted by CF Foundation recommended that patients over 10 years should undergo a 2 h oral glucose tolerance test (OGTT) every year to check for CFRD.[12] Only a few of these patients follow these guidelines, as the reported screening rates are less than 50%.[13] Therefore, adherence to these guidelines could be enhanced by identifying those at the highest risk for developing CFRD early.

Metabolomics is a highly effective analytical method for studying metabolites, a class of small molecules found in cells, biofluids, tissues, and organisms, including amino acids, lipids, and carbohydrates.[14] Comprehensive information about metabolic processes is provided by global metabolomics, which has implications for the identification of biomarkers and the potential explanation of disease pathogenesis.[15,16] Plasma metabolic alterations in CFRD are yet not completely understood, and limited studies have explored the metabolic differences between CFRD and without CFRD (nCFRD) patients.[16] Additionally, the absence of biomarkers linked with impaired glucose metabolism makes it difficult to diagnose CFRD. Therefore, this study aims to identify novel metabolomics biomarkers between CFRD and nCFRD.

MATERIALS AND METHODS

Study Design and Sample Collection. CF patients (n = 36) and age- and gender-matched healthy controls (n = 36) were enrolled in this study. Among 36 patients with CF, nine patients had CFRD. The institutional review board at King Faisal Specialist Hospital and Research Center (KFSHRC) approved this study (RAC# 2160 031). The exclusion criteria were patients who participated in other clinical studies in the last 30 days and were unable or unwilling to provide informed consent. The samples were collected as previously described.[17] In brief, blood samples were taken from adult CF patients who visited the adult CF-Pulmonology clinic at the King Faisal Specialist Hospital and Research Center (KFSHRC) in Riyadh, Saudi Arabia. The samples were centrifugated to separate the serum and frozen at −80 °C for further analysis.

Sample Preparation and Metabolite Extraction. A standard procedure was used for metabolite extraction.[18] Briefly, metabolites were extracted from plasma samples by adding an extraction solvent of ACN:MeOH (1:1) followed by vortexing at 600 rpm, 4 °C for 1 h in a Thermomixer (Eppendorf, Germany). The samples were then centrifuged at 16,000 rpm for 10 min at 4 °C. Subsequently, the supernatant was dried using a SpeedVac (Christ, Germany) and resuspended in 100 μL of 50% A:B mobile phase before LC/MS analysis (A: 0.1% formic acid in dH2O, B: 0.1% FA in 50% ACN:MeOH). Pooled QC was prepared from all samples to check the instrument performance.

LC−HRMS Metabolomics. Untargeted metabolomics analyses were performed using LCMS as previously reported.[19] A Waters Acquity UPLC system coupled with a Xevo G2-S QTOF mass spectrometer equipped with an electrospray ionization source (ESI) was used. The samples were separated by an ACQUITY UPLC and eluted through an XSelect (100 × 2.1 mm 2.5 mm) column (Waters Ltd., Elstree, UK). The composition of mobile phase solvent A was 0.1% formic acid in dH_2O, and that of solvent B was 0.1% formic acid in 50% ACN:MeOH. The flow rate was at 0.300 μL/min. The gradient started with 95%−5% A for 16 min followed by 4 min of 5%−95% A, 1 min of 5%−95% A, and then 2 min of 95−5% A. The total run time was 23 min with an injection volume of 5 μL. MS spectra were acquired under positive and negative electrospray ionization modes (ESI+, ESI−). MS conditions were as follows: the source and desolvation temperatures were set at 150 and 500 °C (ESI+) or 140 °C (ESI−), respectively. The capillary voltage was 3.20 kV (ESI+) or 3 kV (ESI−), the cone voltage was 40 V, the desolvation gas flow was 800.0 L/h, and the cone gas flow was 50 L/h. The collision energies of low and high functions were set off at 10 and 50 V, respectively, in MSE mode. Data were collected in continuum mode with a MasslynxTM V4.1 workstation (Waters Inc., Milford, MA, USA).

Statistical and Data Analysis. The raw data were processed by a standard pipeline using the Progenesis QI v.3.0 software from Waters (Waters Technologies, Milford, MA., USA). Processing started from alignment based on the m/z value and retention time followed by peak picking and signal filtering based on peak quality. Further analyses were conducted on features detected in at least 80% of the samples. MetaboAnalyst version 5.0 (McGill University, Montreal, Canada) (http://www.metaboanalyst.ca, accessed on 5 May 2023) was used for multivariate statistical analysis. The data sets were mean-normalized, Pareto-scaled, and log-transformed to preserve their normal distribution and correctly select the appropriate statistical model. Partial least-squares-discriminant analysis (PLS-DA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) models were created using the normalized data sets. The model's fitness (R2Y) and predictive ability (Q2) values of the OPLS-DA models were validated using permutation validation of 100 samples. Permutation validation of 100 samples was used to evaluate the fitness of the OPLS-DA models (R2Y) and predictive ability (Q2). Univariate analysis was performed using Mass Profiler Professional software (Agilent, Santa Clara, CA, USA). Volcano plots were used to identify significant mass features based on a fold change (FC) cutoff of 2 and false discovery rate (FDR)-corrected p-value < 0.05. Pathway analysis and receiver operating characteristic (ROC) curves with PLS-DA as classification and feature ranking method were created in the MetaboAnalyst v 5.0 for global analysis to evaluate potential biomarkers. The significant features were annotated in Progenesis IQ software and identified based on their exact masses and fragmentation pattern by Human Metabolome Database (HMDB).

RESULTS

Demographics and Clinical Features in Patients with CFRD. Table 1 summarizes the clinical and biochemical

https://doi.org/10.1021/acsomega.4c03626
ACS Omega 2024, 9, 32873−32880

Table 1. Demographic and Clinical Characteristics of CF Patients (n = 36) and Healthy Controls (n = 36)[a]

sample		number	male %	average age	p-value	FEV1 L (%) average
CF	CFRD	9	22%	17.5 ± 5.5	0.23	51% ± 28.3
	nCFRD	27	52%	21.5 ± 5.4		51% ± 25.6
healthy control		36	44%	22.6 ± 9.1		

[a]CFRD: Cystic fibrosis-related diabetes; FEV1: forced expiratory volume in second.

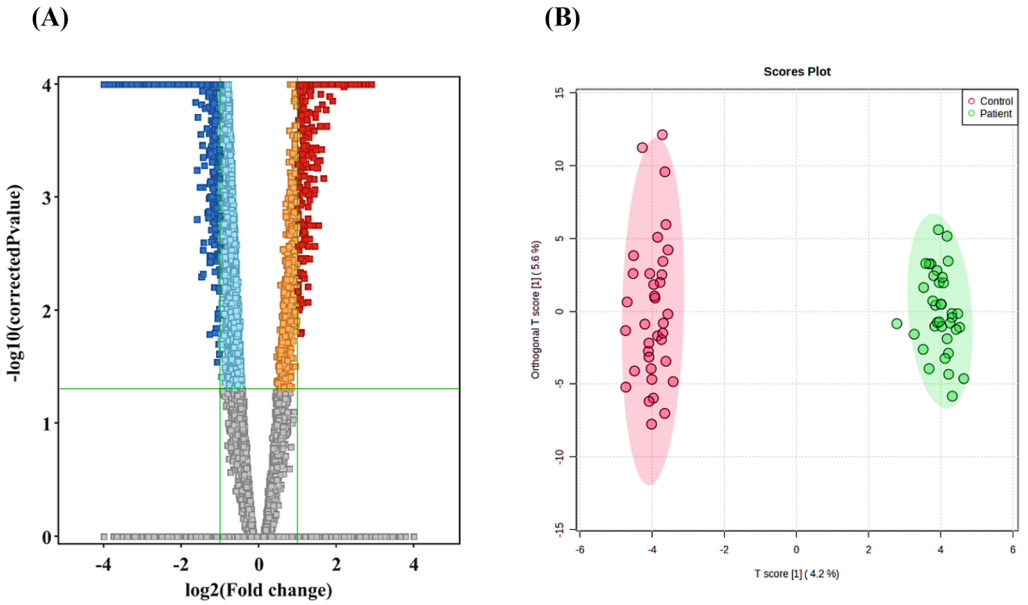


Figure 1. (A) Volcano plot showing significantly dysregulated metabolites between CF and healthy control (modified t test, FDR p-value ≤0.05, fold change (FC) 2). It showed 2328 significantly dysregulated metabolites. 605 metabolites were up-regulated (red) and 1723 were down-regulated (blue) in CF. (B) OPLS-DA shows a clear separation between the two groups. The robustness of the created models was evaluated by the fitness of the model (R2Y = 0.99) and predictive ability (Q2= 0.755) values in a larger data set (n = 100).

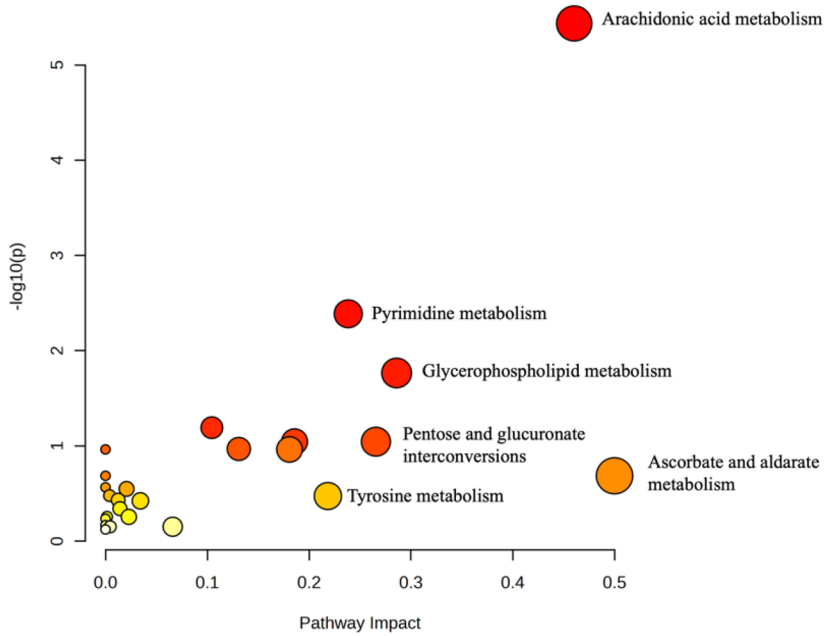


Figure 2. Pathway analysis of 405 significantly dysregulated endogenous metabolites in CF patients showed altered arachidonic acid metabolism and ascorbate and aldarate metabolism. The node color and size are based on the p-value and the pathway impact value, respectively.

information on CF patients. CF patients were 20 females (55.56%), and 16 males (44.44%) with an average age of 20.5 ± 5.6 years, and healthy controls were gender-matched, with an average age of 22.6 ± 9.1 years. The group summary of CFRD (n = 9) and those without CFRD (nCFRD) (n = 27) with their corresponding controls is shown in Tables S1−S3.

Metabolomics Profiling between CF and Control. A total of 51,298 mass ion features were detected in both positive

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ACS Omega 2024, 9, 32873−32880

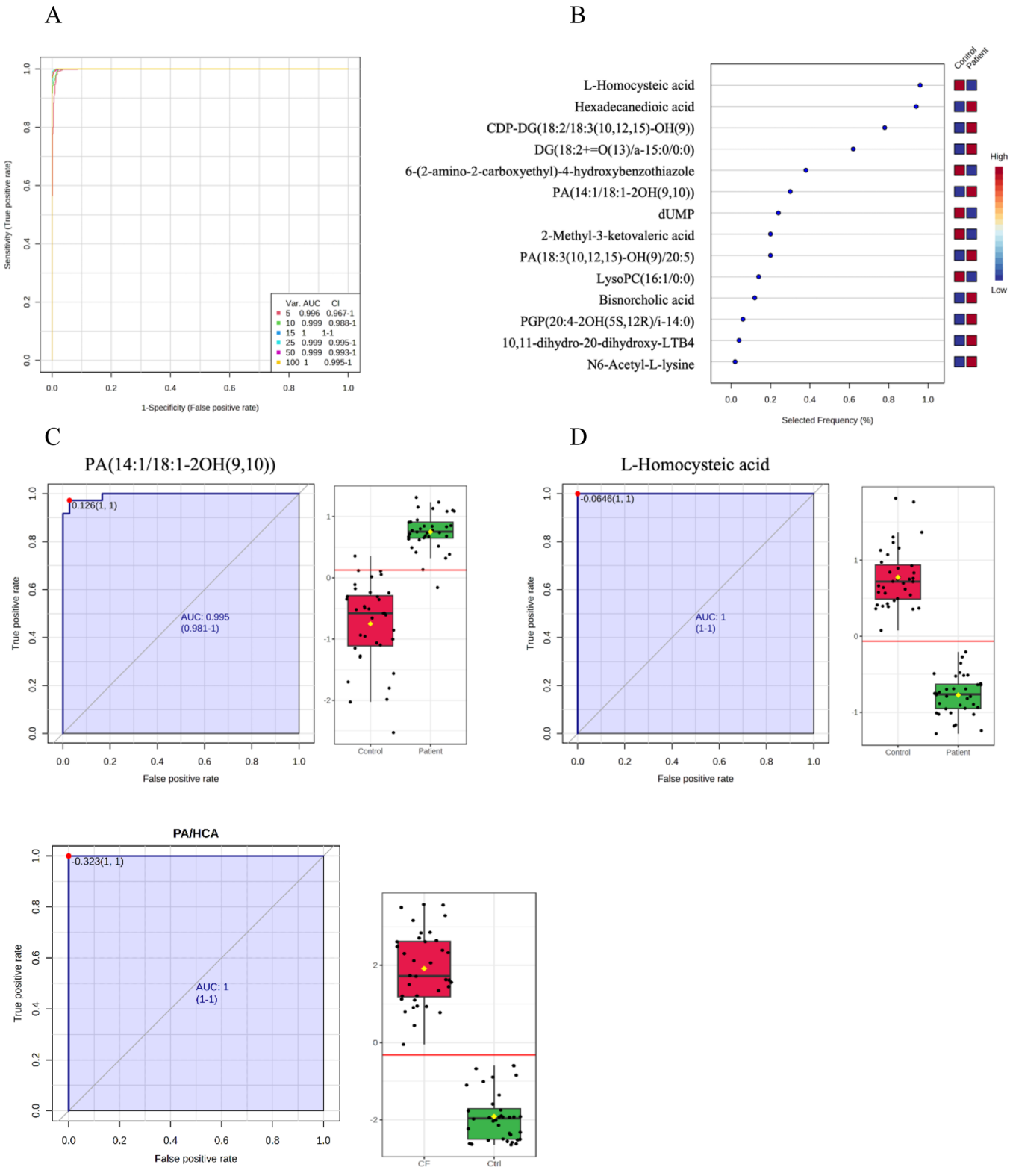

Figure 3. Receiver operating characteristics (ROC) curve and loading for significantly altered metabolites in CF patients and healthy controls. (A) The PLS-DA model produced an ROC with an area under the curve (AUC) of 0.996 for the top 5 variants. (B) Frequency plot ranking the most important features of a selected model. (C) PA (14:1/18:1−2OH(9,10)) with AUC: 0.995 and (D) L-homocysteic acid with AUC: 1, which were upregulated and downregulated in CF patients, respectively. (E) PA (14:1/18:1−2OH(9,10)/HCA ratio with AUC:1, which was upregulated in CF patients compared Ctrl.

and negative ionization modes. Features with missing values of more than 80% of the samples in any group were excluded (51.6%), and the remaining features (24793) were further statistically analyzed. Data were normalized to the sample median, transformed to log 10, and scaled with the Pareto method to ensure all the analytes and samples were normally

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distributed. Univariate analysis was performed to identify significantly different features among the study groups. Then, 24,793 features were statistically evaluated among the two groups using volcano plot analysis (moderated *t* test, FDR *p*-value ≤0.05, FC 2), showing 2328 significantly dysregulated metabolites. A total of 605 metabolites were upregulated, and 1723 were downregulated (Figure 1A). OPLS-DA shows a significant difference between the two groups (CF and healthy control) with R2Y = 0.992 and Q2= 0.755 (Figure 1 B), indicating a significant metabolic difference between the CF and healthy groups. Figure 2 shows the pathway analysis of identified endogenous metabolites, which was performed to determine the most altered pathways. Arachidonic acid (AA) metabolism and ascorbate and aldarate metabolism were among the pathways that had the highest impact.

Biomarker analysis was performed to evaluate potential biomarkers using the receiver operating characteristic (ROC) curve with PLS-DA as the classification and feature ranking method. Figure 3A displays a multivariate exploratory ROC analysis with the top 5 features having an AUC = 0.996. The frequency plot displaying the top 15 important features of a selected model is shown in Figure 3B. The ROC curve of individual biomarkers, PA (14:1/18:1−2OH(9,10)) (AUC = 0.995), L-homocysteic acid (L-HCA) (AUC = 1), and PA (14:1/18:1−2OH(9,10)/HCA ratio (AUC:1) are illustrated in Figure 3C,D,E respectively.

Metabolomics Profiling between CFRD and nCFRD. Univariate analysis using FDR < 0.05 revealed that 799 metabolites were altered between CFRD and nCFRD groups. Volcano plots applying *p*-value and fold change (FC) thresholds of 0.05 and 2, respectively, showed that 44 and 755 metabolites were significantly up- and downregulated, respectively, in CFRD compared to nCFRD (Figure 4).

The identification of metabolites with the potential to serve as prospective biomarkers and the assessment of the diagnostic precision of these metabolites were performed using ROC analysis. PLS-DA was employed as a classification and feature ranking technique to create a multivariate exploratory ROC analysis. Ten features at the exploratory ROC curve had an area under the curve (AUC) value of 0.620 (Figure 5A). Ribothymidine, gamma-glutamylglycine, and L-HCA (Figure 5B) were significantly dysregulated between the study groups with the highest frequency. Gamma-glutamylglycine was upregulated in CFRD compared to nCFRD with an AUC of 0.716 (Figure 5C). In contrast, hydroxy-L-tryptophan was downregulated in CFRD compared to nCFRD with an AUC of 0683 (Figure 5D).

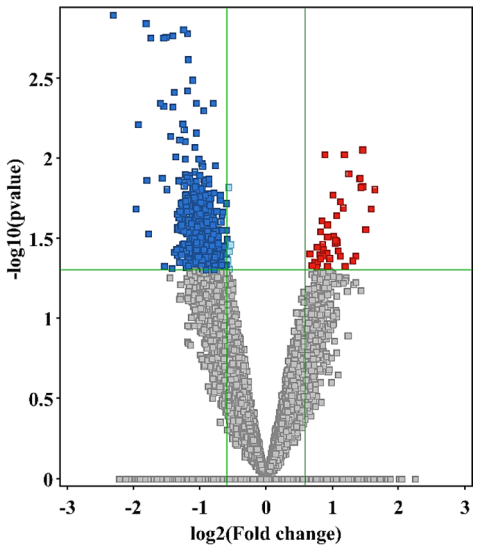

Figure 4. Volcano plot showing significantly dysregulated metabolites between CFRD vs nCFRD (moderated *t* test, *p*-value ≤0.05, fold change (FC) 1.5). It showed 799 significantly dysregulated metabolites. 44 metabolites were upregulated (red) and 755 were downregulated (blue) in CFRD.

DISCUSSION

CFRD is an extrapulmonary complication that increases morbidity and death. Patients with CF and impaired glucose metabolism must be diagnosed early with CFRD, thereby reducing pulmonary decline rates. Therefore, we utilized a metabolomics technique based on mass spectrometry for metabolomics profiling of CFRD and nCFRD, highlighting changed pathways and identifying possible biomarkers that might guide therapy and help in pulmonary deterioration.

AA is a notably altered mechanism between CF and the control. AA is a fatty acid required for synthesizing prostaglandins, thromboxanes, and leukotrienes. It plays a significant role in various physiological processes, including inflammation and immune responses.[20] In the context of CF, consistent with our study, Wheelock et al. found that an increased release of AA in CF is associated with worsening pulmonary symptoms.[21] Moreover, alterations in AA enhance airway mucus production in patients with CF.[22]

Phosphatidic acid (PA) is a key molecule in the production of phospholipids and in the formation of cellular membrane formation. It may also be a key second messenger in several cellular signaling events, such as inflammation.[23] It was found that different CF genotypic classes have a significant impact on PA blood levels.[24] In addition, lung surfactant synthesis and composition may be altered by elevated PA levels, which may affect lung function and cause respiratory distress.[25]

In CF, PA can turn on different inflammatory pathways, such as the nuclear factor-kappa B (NF-B) signaling system, which makes molecules that cause inflammation.[26] This could cause damage to lung cells and can be associated with pulmonary complications.

Our results show that ribothymidine, which is a methyluridine with a single methyl group at the 5-position of the uracil ring, is significantly higher in CFRD compared to nCFRD. This result is consistent with previous results, which showed high concentrations in patients with T2DM reduced by the ani-diabetic effector of berberine treatment.[27]

We have found that homocysteic acid (HCA) levels decreased significantly in both CF compared to the control group and CFRD compared to nCFRD. Oxidation of homocysteine results in the formation of HCA. Furthermore, superoxide oxidation of methionine results in its formation. As a result, HCA is produced due to an increase in homocysteine and via a direct synthesis process. Low HCA in patients with CF and CFRD compared to control and nCFRD, respectively, may be explained due to several amino acids depletion and impaired metabolism.[28] Since HCA is lower in CF compared to healthy controls, and between patients with CF HCA was lower in CFRD than nCFRD, we can suggest the presence of lower HCA may imply either a worse phenotype of CF (i.e., CFRD) or more progression of CF. Low dietary intake and impairment in folate and vitamin B12 absorption will result in

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ACS Omega 2024, 9, 32873−32880

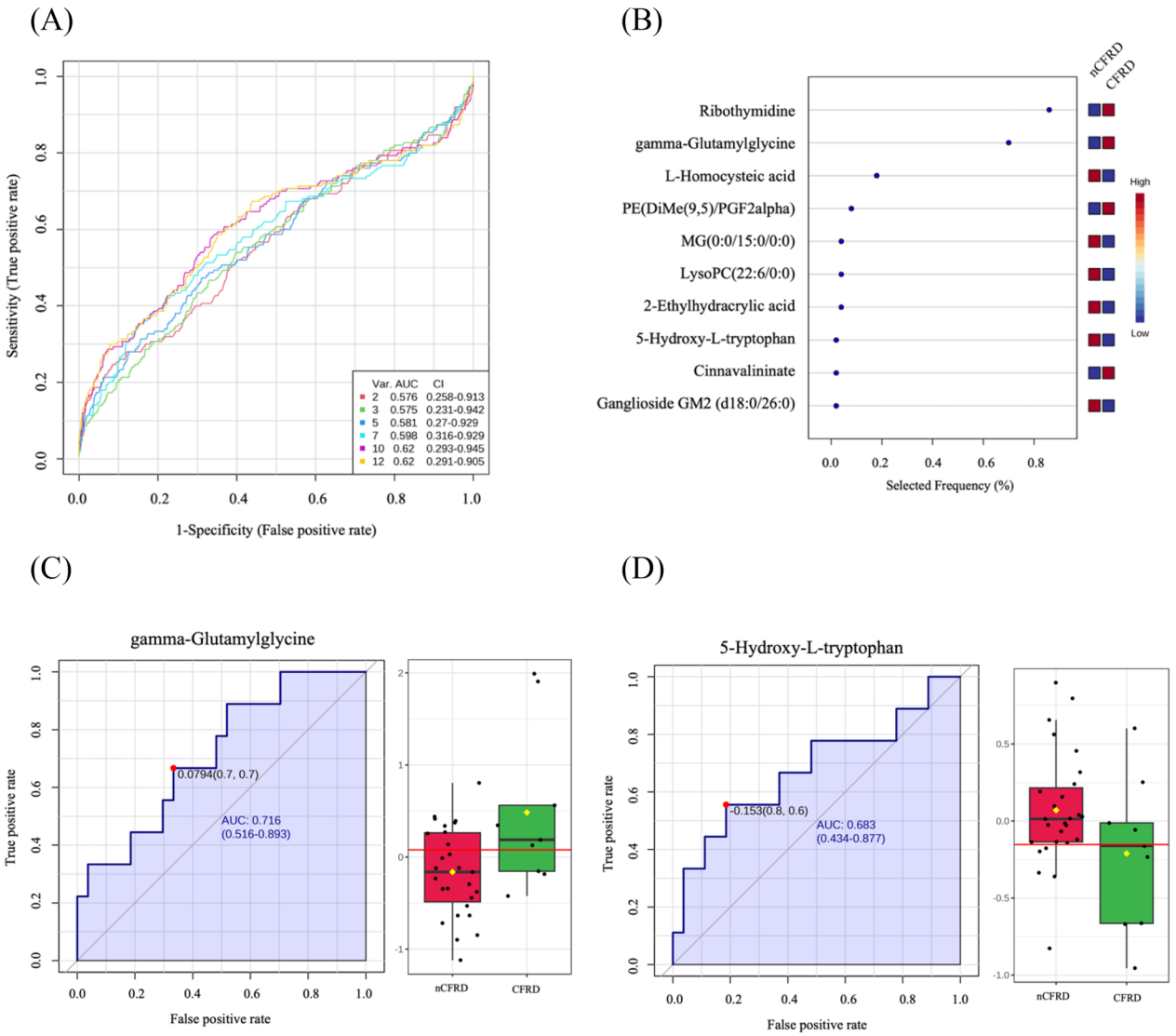

Figure 5. (A) Receiver operating characteristic (ROC) curve based on 12 significantly dysregulated endogenous metabolites between CFRD and nCFRD using PLS-DA as the classification and feature ranking method with an area under the curve (AUC) of 0.581 for the top 5 variants. (B) Frequency plot showing the top 10 significantly dysregulated identified endogenous metabolites. (C) Gamma-glutamylglycine with AUC: 0.716 and (D) 5-hydroxy-L-tryptophan with AUC: 0.683, upregulated and downregulated in CFRD, respectively.

low methionine levels, ultimately inhibiting homocysteine formation and reducing HCA levels.[29]

Gamma-glutamyl dipeptides are a group of bioactive peptides with gamma-glutamyl residues and amino acids. They can be synthesized through the metabolism of Gamma-glutamyl-cysteine synthetase (-GCS) and Gamma-glutamyl transferase (-GGT) in humans and microorganisms.[30] Recent studies have shown that gamma-glutamyl dipeptides are involved in various biological processes. By activating calcium-sensing receptors (CasR) in several organs, these mechanisms include modulation of inflammation, oxidative stress responses, and glucose metabolism.[31] Gamma-glutamyl dipeptide imbalances have been linked to several illnesses, including obesity, MetS, type 2 diabetes, and CVDs.[32] In our study, gamma-glutamyl-glycine was significantly higher in our CFRD compared with non-CFRD, which may be explained by reduced activity of glutamyl transferase, which may reflect the heightened redox and oxidative state caused by CF, and which also concurrently leads to altered levels of glutamate, as was shown in a previous study by et al. Masood.[15] Moreover, gamma-glutamyl dipeptides were associated with incidents of T2DM.[33] Given the pivotal role of oxidative stress in driving the development of complications in diabetes,[34] the formation of gamma-glutamyl dipeptide could serve as a useful indicative biomarker for the complications of CF toward CFRD.

L-5-hydroxytryptophan (5-HTP) is produced by tryptophan hydroxylase (TPH) and decarboxylated to produce serotonin, monoamine neurotransmitters that regulate mood, cognition, reward, learning, memory, sleep, and many other physiological processes.[35] In a mouse model, the buildup of serotonin in the liver but not the brain is linked to the hypoglycemia caused by 5-HTP.[36] When blood sugar levels are high, serotonin triggers insulin release, which has antidiabetic properties.[37] 5-HTP depletion as a precursor to serotonin leads to low levels of serotonin production, and it may indirectly be associated with hyperglycemia, which is consistent with our result.

Consistent with our findings regarding significantly lower levels of lysophosphatidylethanolamine in CFRD compared with nCFRD, a study investigating the correlation between changes in plasma metabolites and the possibility of developing

https://doi.org/10.1021/acsomega.4c03626
ACS Omega 2024, 9, 32873−32880

T2DM over a 10-year period found that lysophosphatidylethanolamine was one of the top three low T2DM risk-associated 10-year metabolite changes.[38]

The metabolite 2-ethylhydracrylic acid is significantly reduced in CFRD compared to nCFRD. This metabolite is produced from isoleucine metabolism.[39] Our findings are consistent with previous research showing that isoleucine metabolism is disturbed due to insulin resistance and with lower insulin production, which has an effect on both T1DM and T2DM.[40]

While this study provides valuable insights, a limitation is the relatively small size of the patient population, which did not fully consider the genetic variations of CF. Future research should expand the study size and incorporate analysis based on different CF-causing mutations to obtain a more comprehensive understanding.

CONCLUSION

To obtain novel insights into the disturbed biochemical pathways in CF and CFRD compared to the control and to find potential biomarkers, the MS-based metabolomics technique was applied to serum samples from patients with CF for the first time to our knowledge. Some of the metabolic pathways most often affected in CF were AA, ascorbate, and aldarate metabolism. The gamma-glutamylglycine and 5-HTP showed the most significant ability to differentiate between CFRD and nCFRD. Moreover, L-HCA levels were significantly reduced in CF and CFRD compared to those in control and nCFRD, respectively. However, these biomarker panels must be verified in a larger multicenter experiment before being utilized in clinical practice.

ASSOCIATED CONTENT

Data Availability Statement

The raw data of this study were deposited to Metabolomics Workbench (https://www.metabolomicsworkbench.org (Released Date September 1, 2023) and can be accessed at (accession number ST002811)

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c03626.

Table S1: Summary of CFRD patients and control; Table S2: Summary of CF(T2DM) patients and control; Table S3: summary of CF (nT2DM) patients and control; Table S4: Raw data of CF patients and healthy controls.; Table S5: Binary comparison of CF patients and healthy controls. (Moderated t.test, p value <0.05, Fold change 2); Table S6: Endogenous metabolites. Moderated t.test, p value <0.05, FC 2; Table S7: Binary comparison of CF patients (T2DM vs nT2DM) on 2328 significant metabolites. (Moderated t.test, p value <0.05, Fold change 2) (XLSX)

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Funding

This research was funded by the University Diabetes Center, King Saud University.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to the University Diabetes Center, King Saud University, for funding this project.

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https://doi.org/10.1021/acsomega.4c03626
ACS Omega 2024, 9, 32873−32880