



Distinctive metabolic profiles between Cystic Fibrosis mutational subclasses and lung function

Afshan Masood¹ · Minnie Jacob² · Xinyun Gu³ · Mai Abdel Jabar³ · Hicham Benabdelkamel¹ · Imran Nizami⁴ · Liang Li³ · Majed Dasouki² · Anas M. Abdel Rahman^{2,5,6}

Received: 13 September 2020 / Accepted: 9 December 2020 / Published online: 4 January 2021
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC part of Springer Nature 2021

Abstract

Introduction Cystic fibrosis (CF) is a lethal multisystemic disease of a monogenic origin with numerous mutations. Functional defects in the cystic fibrosis transmembrane conductance receptor (CFTR) protein based on these mutations are categorised into distinct classes having different clinical presentations and disease severity.

Objectives The present study aimed to create a comprehensive metabolomic profile of altered metabolites in patients with CF, among different classes and in relation to lung function.

Methods A chemical isotope labeling liquid chromatography-mass spectrometry metabolomics was used to study the serum metabolic profiles of young and adult CF (n=39) patients and healthy controls (n=30). Comparisons were made at three levels, CF vs. controls, among mutational classes of CF, between CF class III and IV, and correlated the lung function findings.

Results A distinctive metabolic profile was observed in the three analyses. 78, 20, and 13 significantly differentially dysregulated metabolites were identified in the patients with CF, among the different classes and between class III and IV, respectively. The significantly identified metabolites included amino acids, di-, and tri-peptides, glutathione, glutamine, glutamate, and arginine metabolism. The top significant metabolites include 1-Aminopropan-2-ol, ophthalmate, serotonin, cystathionine, and gamma-glutamylglutamic acid. Lung function represented by an above-average FEV1% level was associated with decreased glutamic acid and increased guanosine levels.

Conclusion Metabolomic profiling identified alterations in different amino acids and dipeptides, involved in regulating glutathione metabolism. Two metabolites, 3,4-dihydroxymandelate-3-O-sulfate and 5-Aminopentanoic acid, were identified in common between the three analyses and may represent as highly sensitive biomarkers for CF.

Keywords Cystic fibrosis · Cystic fibrosis transmembrane conductance regulator · Liquid chromatography-mass spectrometry · Metabolomics · FEV1%

Abbreviations

CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator

Supplementary Information The online version of this article (<https://doi.org/10.1007/s11306-020-01760-5>) contains supplementary material, which is available to authorized users.

✉ Anas M. Abdel Rahman
aabelrahman46@kfshrc.edu.sa

¹ Proteomics Resource Unit, Obesity Research Center, College of Medicine, King Saud University, PO. Box 2925 (98), Riyadh 11461, Saudi Arabia

² Metabolomics Section, Center for Genomic Medicine, King Faisal Specialist Hospital and Research Centre (KFSHRC), Zahrawi Street, Al Maather, PO. Box 3354, Riyadh 11211, Saudi Arabia

³ Department of Chemistry, University of Alberta, Edmonton, AB T6G 2R3, Canada

⁴ Lung Transplant Section, Organ Transplant Center, King Faisal Specialist Hospital and Research Center, Zahrawi Street, Al Maather, Riyadh 11211, Saudi Arabia

⁵ Department of Biochemistry and Molecular Medicine, College of Medicine, Al Faisal University, Riyadh, Saudi Arabia

⁶ Department of Chemistry, Memorial University of Newfoundland, St. John's, NL A1B 3X7, Canada

ROS	Reactive Oxygen Species
VOC	Volatile Organic Compounds
DBS	Dried Blood Spots
GC–MS	Gas Chromatography–Mass Spectrometry
CIL LC–MS	Chemical isotope labeling liquid chromatography–mass spectrometry

1 Introduction

Cystic fibrosis (CF) or mucoviscidosis is a complex, chronic multiorgan autosomal recessive disease that affects the lungs, pancreas (exocrine and endocrine), skin, hepatic system, gastrointestinal, and the urogenital system (Davis et al. 1996) simultaneously. The classical feature of the disease is a malfunction of the exocrine glands resulting in thickening of the normal mucoid secretions that otherwise aid in lubricating the airways and digestive tract. Clogging of the exocrine ducts with viscous mucociliary secretions within the different organs, due to their impaired clearance, forms the basis for the disease's pathophysiology and clinical presentation.

CF's earliest manifestation is observed in neonates who present with meconium ileus and by either wheezing, recurring respiratory infections and pneumonia, steatorrhea, or failure to thrive in children. Although considered a fatal neonatal disease, recent advances in healthcare, diagnosis, and therapies have increased these patients' lifespan into adulthood, albeit with varying clinical presentations. Adult CF patients are commonly present with one or a combination of findings, including; chronic respiratory infections, i.e. bronchiectasis, progressive lung damage, endocrine and exocrine pancreatic insufficiency, malabsorptive gastrointestinal disorders, cirrhosis, osteopenia, and reduced fertility in both men and women (Ratjen et al. 2015). The varying clinical presentation in the adults depends on the type of genetic mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes for a membrane protein and its molecular consequences (Castellani and Assael 2017).

The CFTR protein is a low conductance transmembrane channel protein present on epithelial cells of organs and circulating cells such as neutrophils (Galant et al. 1981; Painter et al. 2006). It belongs to the ATP-binding cassette (ABC) transporters that couples ATP hydrolysis to translate anions via its two nucleotide-binding and membrane-spanning domains. The transmembrane channel so formed helps in chloride and bicarbonate to maintain normal fluid and electrolyte transport across biological membranes. (Li et al. 1996; Schneider and Hunke 1998). More than 2000 variants have been associated with the *CFTR* gene (Maiuri et al. 2017). As per CFTR2, 432 variants of CFTR have been annotated [<https://cftr2.org/>], but not all mutations lead to disease (Castellani et al. 2008). CF is

categorized into different classes based on CFTR protein's dysfunctions and its association with disease pathology or severity (Class I–VI) (Maiuri et al. 2017). Classes I–II, V, and VI disease-causing variants are associated with little or no CFTR function and demonstrate a more severe phenotype. The Class II ($\Delta F508$) phenotype is the most common mutation found among Caucasians.

On the other hand, Class III and IV mutations demonstrate a functionally aberrant CFTR protein and represent a milder form of the disease with preserved lung and exocrine pancreatic function. These mutations are infrequent and show a higher regional or country-specific prevalence (De Boeck et al. 2014). Class III and IV *CFTR* mutations are characterized by anion active ATP binding to the protein or a nonfunctional protein with impaired gating and low conductance, reducing the passage of ions through the channel, respectively (Rogan et al. 2011). These different physiological manifestations of a mutation in a single gene add to the disease's complexity and heterogeneity, underscoring the importance of studying and profiling the perturbations in biochemical and metabolic-related pathways. The CFTR mutational classes connected lipidomics (Zardini Buzatto et al. 2020) and proteomics (Benabdelkamel et al. 2020) profiles have been recently reported in adult CF patients.

Metabolomics is a valuable tool that assesses endogenous metabolites giving a blueprint of metabolic activity related to genetic variations, gene expression, or external stimuli. Global metabolomics provides a complete picture of the metabolisms reflecting on imbalances that may explain the disease's pathophysiology. Metabolomics approaches have been used previously to study differences and identify biomarkers between CF patients and healthy controls using blood, sputum, bronchoalveolar lavage fluid, exhaled breath condensate, and biological urine samples. The majority of these studies focused on identifying metabolites altered with infections, inflammation, disease severity, presence of associated complications, clinical exacerbations, or with destructive lung disease (Muhlebach et al. 2019; Wolak et al. 2009; Esther et al. 2016; Laguna et al. 2015). To date, studies investigating the impact of different CFTR mutational classes on the metabolomics profiles are lacking. In the present study, we have used chemical isotope labeling liquid chromatography–mass spectrometry (CIL LC–MS) approach (Chen et al. 2017) to understand better the basis of the changes in cellular metabolism that result from various *CFTR* mutation classes and on the lung function based on FEV1%.

2 Material and methods

2.1 Chemicals and standards

HPLC grade methanol, chloroform, potassium chloride (KCl), and acetonitrile (ACN) were purchased from Fisher (USA). Metabolite standards and reagents were obtained from Sigma (St. Louis, MO). ^{12}C - and ^{13}C -dansyl chloride were available from www.novamt.com.

2.2 Study design, patient samples, and molecular studies

The study cohort included young and adult patients with a clinical and laboratory-confirmed diagnosis of CF. They were being followed regularly at the adult CF-Pulmonology clinic at King Faisal Specialist Hospital and Research Center (KFSHRC) (Riyadh, KSA) (Al-Qahtani et al. 2020). These patients were randomly selected during regular clinic visits and consented to participate in this study according to the KFSHRC Institutional Review Board (IRB) approved protocol (RAC# 2160 031). A detailed clinical and genetic baseline questionnaire customized for this study and approved by the IRB was completed for each patient (summarized in Table 1) by the recruiting physician from the CF-pulmonology clinic as described in our previous work (Al-Qahtani et al. 2020; Zardini Buzatto et al. 2020). Additional data for CFTR gene mutation analysis have been described previously and presented in Table 2.

Whole blood samples from 69 young adult patients (CF patients ($n = 39$) and healthy controls ($n = 30$) were collected in plain non-EDTA tubes (Vacutainer, BD Biosciences, San Jose, CA, USA), centrifuged, and the serum stored at $-80\text{ }^{\circ}\text{C}$ until further analysis. The healthy age and BMI matched controls were selected from the biobank of the KFSHRC (Riyadh, KSA) blood bank. The details of the patient characteristics, CFTR gene mutation

Table 2 Classification of CFTR mutation based on protein function

CFTR Classes	No. of patients	Age (Avg $\pm$ SEM)	Gender Female (%)	Class (%)
Class II	4	21.5 $\pm$ 1.06	13	10
Class III	14	20.4 $\pm$ 0.944	35	36
Class IV	15	22 $\pm$ 0.73	30	38
Class V	4	20 $\pm$ 0.353	13	10
Class VI	2	23.5 $\pm$ 7.5	9	5

Class II- mutation affecting CFTR protein processing, Class III- gating, Class IV- conductance, Class V- protein deficiency, Class VI- destabilize the channel in post-ER

analysis, DNA Isolation, PCR amplification of genomic DNA, mutational analysis, and sequencing methods have been described previously. (Al-Qahtani et al. 2020). Any patient enrolled in another clinical study in the last 30 days, unable or unwilling to provide informed consent, or diagnosed with conditions other than CF was excluded from this study.

2.3 LC-MS

The CIL LC-MS metabolomics approach was used in this study, as reported before (Dahabiyeh et al. 2020; Jacob et al. 2019). Each sample was labeled with ^{12}C -dansyl chloride (DnsCl), while a pooled sample was generated by mixing all individual samples then labeled by ^{13}C -DnsCl (Guo and Li 2009). The ^{13}C -labeled pooled sample served as a reference for all the ^{12}C -labeled individual samples. Each sample was normalized before LC-MS analysis. LC-UV quantitation was performed to determine the total concentration of dansyl-labeled metabolites (Wu and Li 2012). Each ^{12}C -labeled sample was mixed with the same mole amount of ^{13}C -labeled pooled sample and injected into LC-MS. All labeled metabolites were identified as peak pairs on mass spectra, and the peak area ratios were used for quantitative metabolomic analysis. The labeled samples were analyzed using a Thermo Fisher Scientific Dionex Ultimate 3000

Table 1 Clinical and molecular features in patients with cystic fibrosis mapped with the CFTR mutation classes

Symptoms	No. of patients	CF total	Class II	Class III	Class IV	Class V	Class VI
Persistent cough	34	87%	9%	35%	41%	9%	6%
Lung infections	3	8%	33%	33%	0%	0%	33%
Sinusitis	6	15%	17%	67%	0%	17%	0%
Bowel irregularities	1	3%	0%	0%	0%	100%	0%
CFTR mutations							
Missense	18	46%	2	4	12	0	0
Deletion	7	18%	2	2	1	0	2
Frameshift	9	23%	0	9	0	0	0
Splicing	5	13%	0	0	0	5	0

UHPLC System (Sunnyvale, CA) linked to a Bruker Maxis II quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker, Billerica, MA).

2.4 Data collection, processing, and analysis

Data generated from multiple runs were aligned together based on the peak's accurate mass and retention time. Initially, CSV files (BrukerDaltonics Data Analysis 4.3 Software) were generated from LC–MS spectra, and IsoMS extracted peak pairs (Zhou et al. 2014). Meanwhile, the redundant pairs (e.g. those of Na^+ , NH_3^+ adduct ions, and dimers) were filtered out, and the missing values in the aligned file were filled by Zerofill software (Huan and Li 2015). Before missing value imputation, the zero-filling program can retrieve a significant portion of missing data based on their signals in the raw file (Huan and Li 2015). The peak pairs with 20% or more missing values in the samples within a group were filtered out. Thus, only the peak pairs with consistently detectable ratios ($> 80\%$ of samples) were subjected to missing value imputation. The samples with no ratio value for a given peak pair miss either ^{13}C -labeled and ^{12}C -labeled peaks or one of the peaks within the peak pair. We applied in-house developed algorithms to fill in the missing values in these different cases. In the case of missing both peaks, which were likely caused by ion suppression of an already low-abundant peak pair to be below the ion detection threshold, the average of the peak pair ratios experimentally measured in other samples was replaced the missing value. In the case of missing either the ^{13}C -labeled peak or the ^{12}C -labeled peak in a peak pair for a given sample, which would indicate this peak intensity was below the detection limit for this sample, a detection threshold value was used to replace the missing ^{13}C -labeled or ^{12}C -labeled peak intensity to calculate the $^{12}\text{C}/^{13}\text{C}$ -peak ratio.

A fold-change criterion of greater than 1.5 or less than 0.67 with a false discovery rate (FDR) adjusted p-value less than 0.05, a univariate analysis (volcano plot) was performed for each binary comparison to identify significantly differentially expressed metabolites. The x-axis, on the volcano plot, represents the fold change (FC) between two comparison groups, and the y-axis represents the p-value. Multivariate analyses, including principal component analysis (PCA), partial least square-discriminant analysis (PLS-DA), and Orthogonal Projections to Latent Structures Discriminate Analysis (OPLS-DA) were made using MetaboAnalyst v4.0 (Chong et al. 2018). The metabolites were positively identified by searching against DnsID Library (www.mycompoundid.org) using retention time and accurate mass (Huan et al. 2015). Putative identification was performed by searching accurate mass against MyCompoundID library, which

contains 8021 known human metabolites and 375,809 predicted metabolites (www.mycompoundid.org) (Li et al. 2013).

For statistical analysis among the groups, analysis of variance (ANOVA) using posthoc Tukey's analysis method, with multiplicity-adjusted p-values, was used for each comparison. This type of analysis seemed best to reduce the probability of making a type 1 error since it supports the testing of pairwise differences due to the unequal group sizes among the experimental and the control groups, as seen in our cohort. Further analysis was performed on GraphPad Prism (version 6.0, Graph Pad software, LA Jolla, CA).

The global data analysis was performed using MetaboAnalyst version 4.0 (McGill University Montreal, Canada). The raw data were normalized to the sample total median to ensure all samples were normally distributed, log-transformed, Pareto scaled. Receiver operating characteristic (ROC) curves of individual metabolites were constructed using MetaboAnalyst (univariate ROC curve analysis). For selecting a panel of two or more top significant metabolites and evaluating the panel's performance for group classification, multivariate ROC curve analysis in MetaboAnalyst with Random Forest classification and feature ranking was used.

Furthermore, to explore the interactions among the identified metabolites and predict their involvement in biochemical pathways, the significantly differentially expressed metabolites identified in this study were interrogated using the Ingenuity pathway analysis (IPA) software (Qiagen, Aarhus, Denmark).

2.5 Bioinformatics and pathway analysis

The list of the significantly identified metabolites was entered into IPA, a web-based software application used for the integration, and interpretation of data derived from 'omics experiments, to determine the functional and biochemical interactions of the significantly different metabolites in the data sets. The core metabolomics analysis was carried out for the different data sets to quickly identify the relationships and pathways relevant to each dataset with a score calculated by the software based on the number of network eligible molecules they contain. A higher score is indicative of a lower probability of finding the observed network by random chance.

3 Results

3.1 Demographics, clinical and molecular features

The present study included 39 young adult patients (59% female) with a mean age of 21.1 ± 5.1 years (Table 1). The

different CFTR mutation classes and their distribution were identified, with the highest percentage of patients belonging to class III and IV (38%, $n=15$ and 33%, $n=13$, respectively) and described in detail (Table S1). The baseline questionnaire showed that the predominant presenting clinical symptoms in the overall CF patients were persistent cough (87%) followed by sinusitis (15%), lung infections (8%), and bowel abnormalities (3%). The class wise distribution of the CFTR genotypes was identified and mapped to their class effect (Table 2). Gender, age, and FEV1% matched at least between Class III and IV, the main mutational classes in this cohort.

3.2 Metabolomics profiling for CF compared to Ctrl

The data were combined and globally analyzed using MetaboAnalyst software version 3.0 (<http://www.metaboanalyst.ca>). The raw data were normalized, transformed, and scaled by a median, log, and Pareto, respectively to ensure that all visualized data are under Gaussian distribution. The comparison between CF and Ctrl groups was demonstrated in OPLS-DA. The separation between the two groups represents the degree of perturbation due to the disease genotype and phenotype through the targeted analytes ($R^2=0.984$, $Q^2=0.962$) (Fig. 1a). The volcano plot analyses were performed on the detected features ($n=2658$). Among them, a group of metabolites ($n=785$) was differentially expressed and visualized in the volcano plot. The cutoff p -value has a corresponding p -value of less than 0.05 and a fold change cutoff value of 1.5. Five hundred and seventy-three detected metabolites were up-regulated, while 212 metabolites were down-regulated in the CF group compared to Ctrl.

Accurate mass and retention time were used to positively identify 259 peak pairs in the database. 35 and 43 metabolites were up- and down-regulated in CF vs. Ctrl, respectively, as shown in the volcano plot (Fig. 1b) and detailed in Table 3. Pathway analysis for the dysregulated metabolites in the CF serum, using MetaboAnalyst, identified aminoacyl t-RNA biosynthesis pathway as the most perturbed, followed by amino acid metabolism pathways arginine biosynthesis, proline, tyrosine, and histidine metabolism pathways as shown in (Fig. 1c) and detailed in Table 4. O-phosphoethanolamine, Cystine, Lysyl-Aspartate, Gamma-Glutamyl-glutamate, 2-hydroxy-3-4 hydroxy, 4-hydroxybenzoic acid, Leucyl-Leucine, and thyroxine were up-regulated in the CF group when compared to the controls (Fig. 1d). Multivariate exploratory ROC analysis with Random Forest classification and feature ranking was used to determine the panels of top significant metabolites in ROC curves (Fig. 1e). Figure 1e shows the AUC of 1 for all panels. The top 5 metabolites panel includes cysteine, O-phosphoethanolamine, Hydroxybenzoic acid, Thyroxine, and Lysyl-aspartate.

3.3 The metabolic pattern among all the CFTR Classes (II–VI)

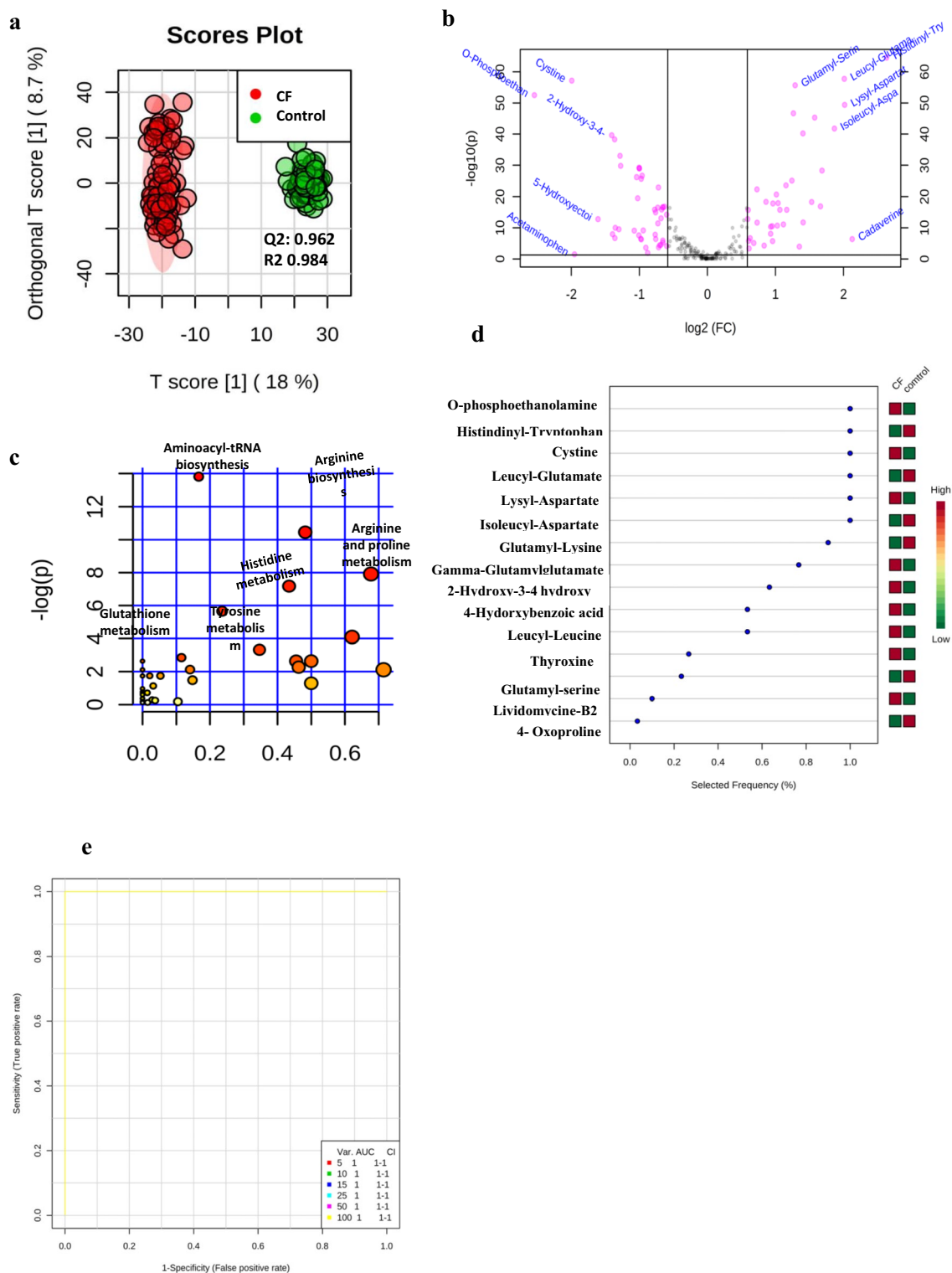
PLS-DA score plot demonstrates a slight separation among the five CF classes (Fig. 2a). These differences between the groups revealed 20 significantly dysregulated metabolites (Table 5) and contributed to the ANOVA test's significance (Fig. 2b). Differential deregulation of the metabolites between the classes of CF was demonstrated. An increase in glutamic acid and propyl glutamate was observed in CF Class II, R-1 Aminopropan-2-ol Ophthamate and tyrosyl-asparagine, 5 aminopentanoic acid in CF Class V and serotonin and 2,5-dihydroxypuridine in class IV as detailed in Fig. 2c. Pathway analysis identified nitrogen metabolism pathways to be the most perturbed, followed by amino acid pathways like the glutamine and glutamate metabolism pathway and arginine biosynthesis pathways when comparisons were made among all the five classes in the CF cohort (Fig. 2d).

3.4 The metabolic pattern for CFTR Class between III and IV

Similarly, the OPLS-DA plot between CF class III and IV also demonstrated a clear cluster separation between the two groups ($R^2=0.978$, $Q^2=0.684$) (Fig. 3a). The volcano plot analyses were also performed, and 13 significantly dysregulated metabolites (6 and 7 metabolites were up and down-regulated respectively) (Table 6) and were visualized in the volcano plot, with fold change > 1.5 or < 0.67 and FDR, p -value < 0.05 (Fig. 3b). The differentially identified metabolites' significant features showed a decrease in R-1-Aminopropan-2-ol, ophthamate, allocystathione, hypoxanthine, 2,5-dihydroproline, alanyl-serine, n-6 acetyl-lysine whereas serotonin was increased in CF class III vs. class IV (Fig. 3c). Multivariate exploratory ROC analysis was used for evaluating group classification performance. The combination of the top metabolites in ROC curves shows AUCs ranging from 0.824 to 0.88 (Fig. 3d). Two metabolites had the highest discriminatory R-1 Aminopropan-2-ol, and ophthamate increased in class IV vs. III with AUC- 0.906 (Fig. 3e) and an AUC- 0.744 (Fig. 3f), respectively.

3.5 The metabolic pattern for lung function of CF patients

A binary comparison was carried out to compare the metabolites altered in patients with an FEV1% above and below the average. The OPLS-DA plot demonstrated clear separation between the patients with an above-average FEV% compared to those with a below-average FEV% (



$R^2=0.988$, $Q^2=0.731$) (Fig. 4a). A volcano plot analysis demonstrated visually the dysregulation of 6 differentially expressed metabolites, with a fold change of >1.5 or <0.67 and an FDR p -value <0.05 (Fig. 4b). Asparaphenylalanine (Fig. 4c), L-2 amino-3-oxobutanoic acid (Fig. 4d), and

Glutamic acid (Fig. 4e) were found to be decreased in the above-average forced expiratory volume in 1 s (FEV1%) group when compared to the below-average FEV% group whereas Histidinyl-tyrosine (Fig. 4f) and Guanosine

Fig. 1 Pathway analysis of the statistically dysregulated metabolites in cystic fibrosis (CF) compared to control (Ctrl). **a** Orthogonal partial least square discriminant analysis (OPLS-DA) score plot shows clear separation due to the biological differences between CF ($n=39$, red dots) and healthy control ($n=30$, green dots) with $R^2=0.984$ and $Q^2=0.962$ that represents the level of global metabolic differential expression. **b** Volcano plots shows statically significant altered metabolites (false discovery rate FDR with fold change >1.5 or <0.75 , $p\text{-value}<0.05$). Pink dots represents features above or below the threshold (fold changes, $p\text{-value}$, $\log_{10}$ and $\log_2$ transformed). **c** Pathway analysis shows significantly perturbed pathways like amino acyl t-RNA biosynthesis and some amino acid to be perturbed based on pathway impact score and $p\text{-value}$. **d** Loading 1 score plots shows significant metabolites like o-phosphoethanolamine, cystine, lysyl-aspartate, gamma-glutamylglutamate to be up regulated in the CF patients compared to controls. **e** ROC curves were generated by multivariate ROC analysis in MetaboAnalyst with Random Forest classification and univariate AUROC feature ranking, showing area under the curve (AUC)=1 for all panels

(Fig. 4g) were found to be increased in the above-average FEV% group.

3.6 Network pathway analysis

The bioinformatics and network pathway analysis using IPA was carried out to demonstrate the dysregulated metabolites' biological significance and relate them to known pathways. The identified metabolites in the data set between patients with CF vs. Ctrl revealed that the dysregulated metabolites altered pathways related to amino acid metabolism, developmental disorder, hereditary disorder, as shown by the highest scoring network pathway (score of 19) (Fig. 5a). The IPA's network score is calculated, taking into account the number of focus metabolites from the data set and the final network size to provide a biologically relevant network relating to the identified metabolites. It is based on the hypergeometric distribution and calculated using the right-tailed Fisher's Exact Test, with the log (Fisher's Exact test result). A score greater than 3 ($p<0.001$) indicates a greater than 99.9% confidence that a protein network was not generated by chance alone (Long et al. 2004; Calvano et al. 2005). The dysregulated metabolites among all the CF patients altered the network pathway related to amino acid metabolism, small molecule biochemistry, cell-to-cell signaling, and interaction (score of 11) (Fig. 5b).

In contrast, the network pathway identified between Class III vs. IV was related to cell-to-cell signaling and interaction, molecular transport, and small molecule biochemistry (score of 14) (Fig. 5c). The nodes with the highest connectivity in the network interaction map for patients with CF demonstrated that the identified metabolites centered around pathways related to the regulation of cyclic AMP, PI3 kinase, ERK $\frac{1}{2}$, Akt, and L-glutamic acid while that of all patients with CF focused around cyclic AMP, L-glutamic acid epidermal growth factor receptor (EGFR). On the other hand,

the network between CF III and CF IV classes showed that alterations in the metabolites centered around deregulation of pathways related to 5-hydroxytryptamine (serotonin), and EGFR.

4 Discussion

Metabolomic alterations due to genetic variations and pathophysiological changes are reflected in the circulating metabolites' changes. In the present study, metabolomic profiling of serum of clinically stable adult patients with CF ($n=39$) in comparison to the Ctrl group ($n=30$) was carried out and identified 78 significantly differentially abundant metabolites altered with the disease regardless of the CF mutational classes. The CF group was next classified according to the different mutational classes (class II-VI) that revealed a panel of 20 significantly dysregulated metabolites between the other classes. We further carried out the analysis between the mutational classes III and IV, representing the milder form of the disease, which showed significant differences in the regulation of 13 metabolites.

Recent studies have shown that CF is not merely a disturbance of the chloride channel or anion conductance but rather a disruption of an extensive network of proteins interacting with CFTR receptor protein for their activity (Gadsby et al. 2006). This indicates that aside from its well-characterized role in regulating chloride ions, it has an expanded role in regulating other proteins and metabolites via different biochemical pathways. In the present study, several amino acids, di-peptides, and tri-peptides were differentially regulated in CF patients, among the different mutational classes, and between Class III and IV.

Two metabolites were found in common among the three analyses (CF vs. control, among CF classes, and between CF III vs. CF IV); namely, 3,4-dihydroxymandelate-3-O-sulfate and 5-Aminopentanoic acid. 3,4-dihydroxymandelate-3-O-sulfate is a norepinephrine metabolite produced explicitly by the mesenteric organs (Eisenhofer et al. 1996). In one study, epinephrine levels were shown to stimulate CFTR, depending on the type of mutations present. An increase in the levels of 3,4-dihydroxymandelate-3-O-sulfate in this group of patients may also act as a compensatory mechanism to increase the activity of CFTR in these patients. 5-Aminopentanoic acid, on the other hand, is a lysine degradation product and a metabolite of cadaverine (Callery and Geelhaar 1984) primarily a microbial metabolite produced by the gut or oral microflora. However, it can be produced endogenously and functions as a weak GABA agonist (Callery and Geelhaar 1985). The differences in the metabolomic patterns in these groups demonstrate that the biological effects due to different mutations of a single gene such as CFTR do not result in the same clinical presentation of the disease but

Table 3 Significantly dysregulated metabolites between Cystic Fibrosis patients (CF) and Healthy control (Ctrl) based on a Volcano Plot analysis that combines T-test and fold change analyses

Name of the metabolite	FC	$\log_2(\text{FC})$	p-value	$\log_{10}(p)$
Histidiny-Tryptophan	6.19	2.63	2.77E-65	64.56
Leucyl-Glutamate	4.01	2.01	1.64E-58	57.78
Cystine	0.25	- 2.00	2.82E-58	57.55
Glutamyl-Serine	2.43	1.28	7.18E-56	55.14
O-Phosphoethanolamine	0.17	- 2.55	5.12E-53	52.29
Lysyl-Aspartate	4.03	2.01	5.99E-50	49.22
4-Oxoproline	2.39	1.26	3.26E-47	46.49
Glutamyl-Lysine	2.97	1.57	7.47E-46	45.13
Isoleucyl-Aspartate	3.63	1.86	2.02E-42	41.69
2-Hydroxy-3-4-hydroxyphenylpropenoate O-glucuronide	0.38	- 1.41	7.57E-41	40.12
Leucyl-Leucine	2.63	1.40	1.40E-40	39.85
4-Hydroxybenzoic acid	0.39	- 1.37	4.20E-39	38.38
Thyroxine	0.41	- 1.29	4.11E-34	33.39
Lividomycin B	0.41	- 1.28	1.21E-30	29.92
4-Hydroxyphenylacetaldehyde	0.50	- 1.01	3.20E-30	29.50
2,4-Dichlorophenol	0.63	- 0.66	5.33E-29	28.27
Gamma-Glutamylglutamic acid	3.21	1.68	5.58E-29	28.25
2R,5S-2,5-Diamino hexanoate	0.51	- 0.97	1.76E-27	26.76
4-Hydroxyphenylacetaldehyde	0.48	- 1.05	3.08E-27	26.51
3S,5S-3,5-Diaminohexanoic acid	0.51	- 0.98	1.78E-26	25.75
Glutamyl-Methionine	2.22	1.15	2.80E-24	23.55
Tyrosine	0.60	- 0.73	4.74E-24	23.32
Lysine	0.65	- 0.63	9.67E-24	23.01
Threoninyl-Glutamate	1.65	0.72	6.70E-23	22.17
Lysyl-Glutamate	2.01	1.00	2.98E-21	20.53
Phenylalanyl-Glutamate	0.49	- 1.03	1.23E-20	19.91
N-Acetyl-L-Asparagine	2.88	1.53	5.44E-19	18.26
N-Acetyloxindoxyl	1.81	0.85	9.38E-19	18.03
Glutamic Acid	2.05	1.03	1.66E-18	17.78
Salicylic acid	1.93	0.95	2.31E-18	17.64
Urocanic acid	0.64	- 0.64	7.29E-18	17.14
Ornithine	0.63	- 0.66	9.64E-18	17.02
Phenylalanyl-Tryptophan	0.63	- 0.66	1.30E-17	16.89
5-Aminopentanoic acid	3.16	1.66	2.15E-17	16.67
Prolyl-Lysine	0.62	- 0.68	6.78E-17	16.17
Glutamyl-Glutamic acid	2.24	1.16	1.88E-16	15.73
3,4-Dihydroxyphenylacetaldehyde	2.08	1.06	2.24E-16	15.65
Arginylglutamic acid	1.51	0.59	3.49E-16	15.46
3-Aminoisobutanoate	0.58	- 0.78	8.12E-16	15.09
(2E)-3-(2,3-dihydroxyphenyl)prop-2-enoic acid	0.66	- 0.61	3.14E-15	14.50
Homoarginine	0.62	- 0.69	7.72E-14	13.11
5-Hydroxyectoine	0.33	- 1.62	1.42E-13	12.85
2-Aminoacrylate	0.60	- 0.73	1.63E-12	11.79
Tyrosyl-Asparagine	2.63	1.39	1.92E-12	11.72
Gentisate aldehyde	1.65	0.72	2.88E-12	11.54
Tyrosyl-Serine	2.16	1.11	7.75E-12	11.11
Phenylalanyl-Threonine	2.02	1.02	2.21E-11	10.66
L-threo-3-Methylaspartate	1.92	0.94	3.81E-11	10.42
Alanyl-Glutamic acid	1.90	0.92	6.09E-11	10.22
3,4-Dihydroxymandelate	0.39	- 1.35	6.95E-11	10.16
Glycyl-Proline	0.40	- 1.31	2.21E-10	9.66

Table 3 (continued)

Name of the metabolite	FC	log ₂ (FC)	p-value	log ₁₀ (p)
Lysyl-Glycine	0.52	− 0.95	4.74E-10	9.32
Aspartylphenylalanine	0.47	− 1.08	5.64E-10	9.25
4-Hydroxy-3-methoxy-benzaldehyde	0.38	− 1.41	9.94E-09	8.00
Hypotaurine	1.89	0.92	1.14E-08	7.94
3–2,3-Dihydroxyphenylpropanoate	0.59	− 0.77	1.15E-08	7.94
Glutamyl-Tyrosine	0.49	− 1.02	2.03E-08	7.69
Histidiny-Tyrosine	0.39	− 1.37	1.82E-07	6.74
Aspartyl-Phenylalanine	0.51	− 0.98	2.70E-07	6.57
Prolyl-Glutamate	1.54	0.63	2.70E-07	6.57
Aspartyl-Lysine	0.59	− 0.77	3.29E-07	6.48
Alanyl-Proline	0.51	− 0.98	4.25E-07	6.37
Cadaverine	4.38	2.13	5.56E-07	6.25
L-gamma-Glutamyl-L-alpha-aminobutyrylglycine	0.63	− 0.66	7.04E-07	6.15
Phenylalanyl-Serine	1.52	0.60	1.60E-06	5.80
p-Hydroxyphenylacetic acid	1.93	0.95	2.39E-06	5.62
Syringic acid	0.63	− 0.66	3.85E-06	5.41
L-2-Amino-3-oxobutanoic acid	1.60	0.67	1.18E-05	4.93
Isoglutamine	0.62	− 0.69	2.62E-05	4.58
Theophylline	0.61	− 0.71	3.50E-05	4.46
Isoleucyl-Threonine	0.66	− 0.60	5.37E-05	4.27
Pipecolic acid	1.77	0.82	6.80E-05	4.17
Tyramine	2.53	1.34	0.000124	3.91
Lysyl-Alanine	0.61	− 0.72	0.000135	3.87
Histidiny-Valine	0.53	− 0.91	0.000203	3.69
3-Aminoisobutanoic acid	1.52	0.60	0.000535	3.27
Vanillic acid	0.54	− 0.89	0.009749	2.01
Acetaminophen	0.26	− 1.96	0.028881	1.54

The cutoff for these two analyses are 0.05 and 1.5, respectively

Table 4 Statistically significant pathways in CF patients in comparison with control

Pathway Name	Match Status	p-value	−log(p)	Holm p	FDR	Impact
Aminoacyl-tRNA biosynthesis	15/48	1.00E-06	13.813	8.42E-05	8.42E-05	0.16667
Arginine biosynthesis	7/14	2.89E-05	10.452	0.0024	0.00121	0.48223
Arginine and proline metabolism	10/38	3.65E-04	7.9154	0.02994	0.01022	0.6771
Histidine metabolism	6/16	7.64E-04	7.1766	0.0619	0.01605	0.43442
Tyrosine metabolism	9/42	0.00349	5.6568	0.2795	0.05869	0.23594
Alanine, aspartate and glutamate metabolism	6/28	0.01673	4.0905	1	0.23424	0.621
Cysteine and methionine metabolism	6/33	0.03589	3.3272	1	0.43072	0.34663

rather in distinct phenotypes that differ in their biochemical changes and disease presentations (Marson et al. 2016).

4.1 Metabolite and pathways altered between CF and Ctrl

The top metabolites increased in the CF vs. controls were O-phosphoethanolamine, cystine, lysyl-aspartate, glutamyl glutamic acid, and 4-hydroxybenzoate, while those that

decreased included histidiny-tryptophan, leucyl-glutamate, isoleucyl-aspartate, glutamyl-lysine, glutamyl-serine, and oxoproline (Fig. 1b). The metabolites were grouped based on their involvement in regulating different biochemical pathways for ease. The top three pathways, related to the identified metabolites, using MetaboAnalyst showed their involvement in the regulation of tRNA synthesis, arginine biosynthesis, arginine, and proline metabolism (Fig. 1c).

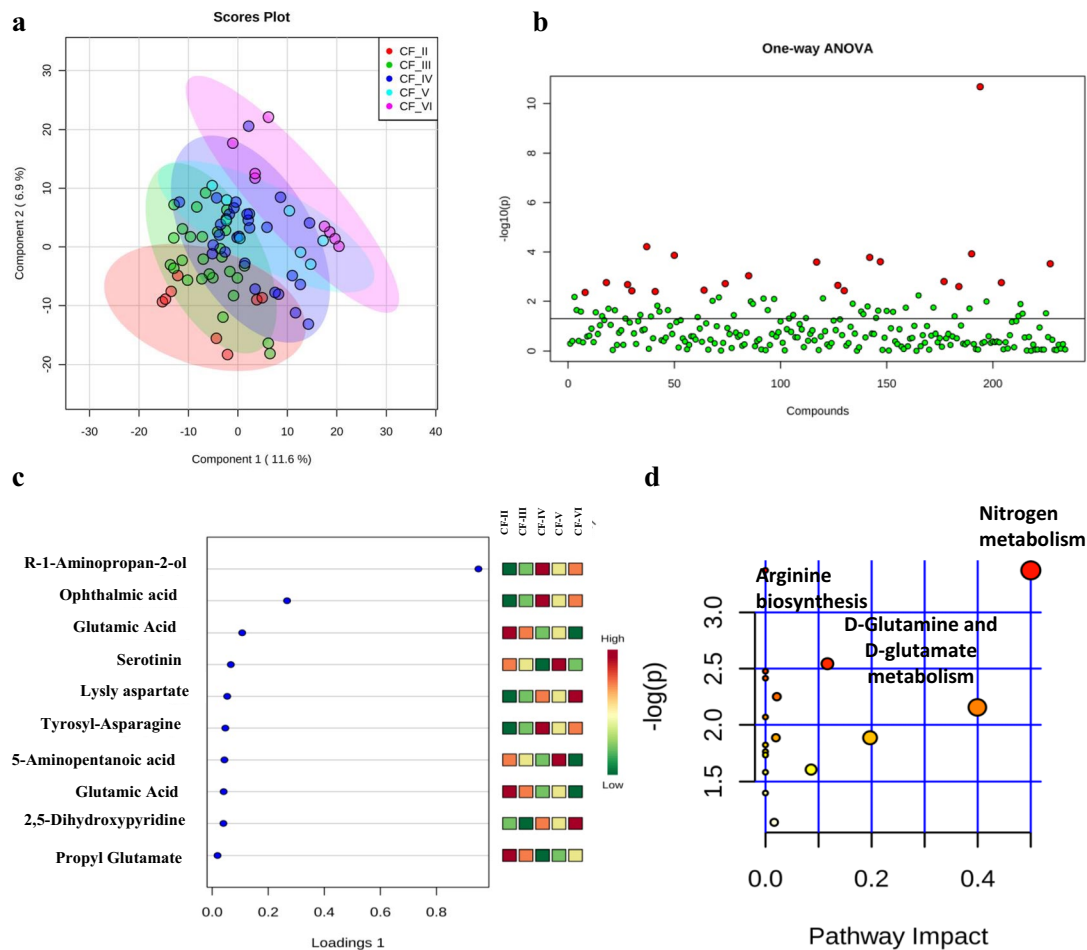


Fig. 2 Pathway analysis and loading plots among five CF classes (II–VI) **a** Overall comparison among the CF classes shows clear separation among the five classes **b** One way ANOVA analysis shows 21 positively identified metabolites among the CF classes shows clear separation among the five classes. **c** Loading 1 score plots show up

regulation of R-1-Aminopropan-2-ol and ophthalmate in class IV CF patients. **d** Pathway analysis among the CF classes shows significantly altered pathways like nitrogen metabolism and some amino acid metabolism pathways

The tRNA charging pathway (Fig. 1c, Table 4) is involved in activating amino acids to incorporate protein synthesis. Patients with CF almost always present with malnutrition, maldigestion/malabsorption of dietary protein due to exocrine pancreatic insufficiency, failure to thrive, and have low muscle mass (Moran et al. 2001). This functional pathway's significant downregulation in patients with CF points to decreased levels of endogenous protein synthesis or anabolic capacity in these patients. The signs of failure to thrive were noted in only one patient as all the others received pancreatic enzyme supplements. The second canonical pathway altered involved biosynthesis of 4-hydroxybenzoate, a tyrosine derivative, and the precursor metabolite responsible for the synthesis of the head group of Coenzyme Q (CoQ). CoQ is a redox-active lipid that plays a critical role in the electron transport chain, oxidative phosphorylation, fatty acid oxidation, uridine biosynthesis, and lipophilic functions

antioxidant in plasma lipoproteins (Stefely and Pagliarini 2017). Three metabolites are identified in our dataset: 4-hydroxybenzoic acid, L-glutamic acid, and L-tyrosine are known to participate in CoQ synthesis. A decrease in 4-hydroxybenzoic acid and L-glutamic acid and an increase in L-tyrosine may indicate decreased hydroxybenzoate synthesis and its related downstream metabolites affecting the energy production antioxidant status of patients with CF.

We found differential regulation of three amino acids involved in arginine biosynthesis. A decrease in levels of ornithine and glutamine and an increase in L-glutamic acid were noted (Table 4, Fig. 1b). Ornithine and glutamine are involved in the urea cycle and are intermediates in the pathway for arginine synthesis. Arginine is an essential substrate for proteins, urea, polyamines, creatinine phosphate, and nitric oxide (NO) synthesis. A decrease in the metabolites of this pathway may indicate decreased

Table 5 Significantly dysregulated metabolites among all Cystic Fibrosis patients (CF) classes, based on a Volcano Plot analysis that combines T-test and fold change analyses

Name of the metabolite	FC	log ₂ (FC)	p-value	log ₁₀ (p)
R-1-Aminopropan-2-ol	20.402	4.91E-09	2.10E-11	10.678
Alanyl-Alanine	7.1639	0.007144	6.11E-05	4.2143
2-Amino-5-oxohexanoate	6.6716	0.007791	0.00012	3.9225
Beta-Alanine	6.5706	0.007791	0.000137	3.8621
Isomer of Desaminotyrosine	6.4316	0.007791	0.000166	3.7787
Seryl-Tyrosine	6.1431	0.008597	0.000249	3.6045
Acetaminophen	6.1188	0.008597	0.000257	3.5898
N2'-Acetyl-3'-Hydroxy-L-kynurenine	6.0075	0.00879	0.000301	3.5222
Isomer of Lysyl-Aspartate	5.2183	0.023891	0.000919	3.0367
Ophthalmate	4.8421	0.034083	0.001578	2.8018
Symmetric dimethylarginine	4.7733	0.034083	0.001743	2.7586
3,4-Dihydroxymandelate 3-O-sulfate	4.7714	0.034083	0.001748	2.7575
Alanyl-Serine	4.6475	0.034546	0.002092	2.7169
Tyrosyl-Asparagine	4.5961	0.03496	0.002254	2.6795
L-2-Amino-3-oxobutanoic acid	4.5187	0.035158	0.002522	2.6471
5-Aminopentanoic acid	4.2878	0.036885	0.003532	2.5982
Glycyl-Histidine	4.2553	0.046284	0.003704	2.452
Glutamic Acid	4.2453	0.046284	0.003758	2.4314
N-Alpha-acetyllysine	4.2016	0.046284	0.004006	2.425
Hypoxanthine	4.143	0.046874	0.004365	2.3973

The cutoff for these two analyses are 0.05 and 1.5, respectively

arginine synthesis in patients with CF. Our findings are in line with other studies showing low levels of circulating L-arginine and deficiency of NO (de Winter-de Groot and van der Ent, 2005) have been previously described in patients with CF. Ornithine serves as an intermediate for L-arginine and a precursor for the formation of polyamines (e.g. putrescine, spermidine, and spermine) and proline, which control cell proliferation and collagen production (Grasemann et al. 2006). The decrease in ornithine and arginine levels was also noted in the dried blood spot metabolomic profile obtained from the neonates with CF (Al-Qahtani et al. 2020). We also identified a significant decrease in the levels of vanillic acid in patients with CF. Makini et al. showed that vanillic acid acted as an antioxidant and was involved in elevating levels of glutathione and other antioxidative enzymes (Makni et al. 2012). A decrease in its levels also points to decreased antioxidant activity in CF. A number of dipeptides of glutamine, lysine, alanine and phenylalanine, a phenol derivative, amines and diamines known to have uncharacterised biological functions were also identified.

All the metabolites identified in the CF patients were interrogated using the IPA analysis to determine the pathways associated with the identified metabolites in our dataset and the disease pathology. The network pathway is highlighted in amino acid metabolism, development disorder, and hereditary disorder connected to the metabolic profile (Fig. 5a). The non-directional interaction map of the

identified metabolites was centered around the phosphoinositide kinase 3 (PI3K) signaling pathway, ERK 1/2 pathway, Akt pathway, cAMP, and glutamate (Fig. 5a). The PI3K signaling pathway's involvement was confirmed through a proteomic study (data not are shown), which demonstrated a significant downregulation in the levels of the protein in CF. The second node showed the involvement of cAMP, which is well-known as the regulator for CFTR. The network pathway also revealed the involvement or deregulation of the NOS pathway between the CF and controls and glutamate, which serves as a significant metabolic hub involved in diverse metabolic processes, including glutathione biosynthesis catabolism of numerous amines (Walker and van der Donk 2016).

Like those enrolled in our study, patients with CF are invariably treated with antibiotics to prevent repeated infections. In line with this, we identified two differentially regulated microbiome-related metabolites, namely, Lividomycin B and syringic acid. Lividomycin B is a secondary metabolite produced by *Streptomyces lividus* that may contribute to the increased antibiotic resistance (Bolourian and Mojtahedi 2018, Ogawara, 2019) patients with CF. Syringic acid is also a microbial metabolite found in *Bifidobacterium* (Boto-Ordóñez et al. 2014), which typically represents the most abundant bacteria of the human gut microbiota. It is also a product of microbial (gut) metabolism of anthocyanins and other polyphenols that have been consumed (in fruits and alcoholic beverages (Forester and Waterhouse 2008).

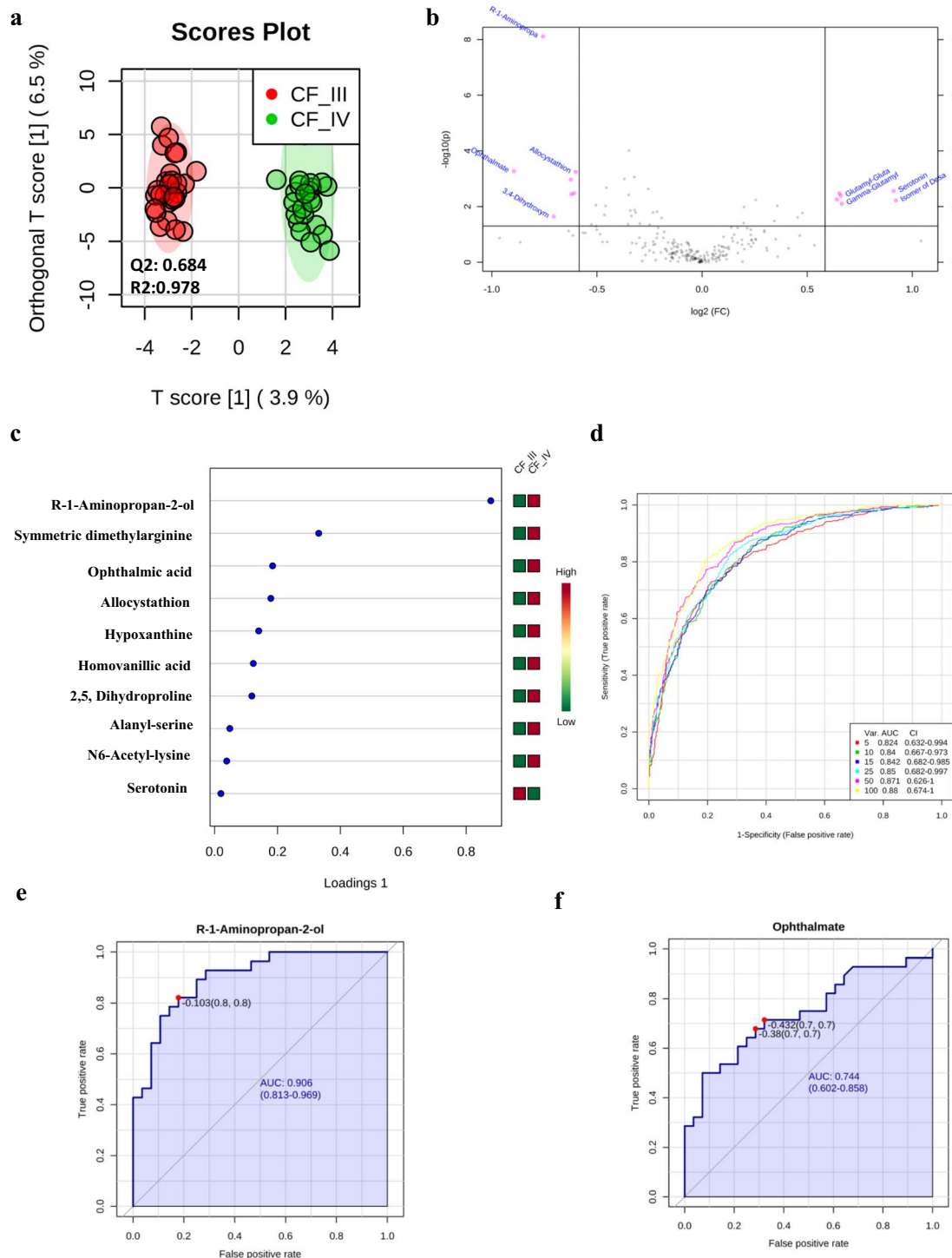


Fig. 3 Differential expression of metabolic profiles in serum of patients with CF classes III vs IV. **a** OPLS-DA plot for binary comparisons between CF classes III ($n = 15$) vs IV ($n = 13$) with a calculated space of $R^2 = 0.978$ and $Q^2 = 0.684$ that represents the level of differential expression of the panel analytes. **b** Volcano plot with fold change > 1.5 and p -value < 0.05 shows 10 metabolites to be significant among class III and IV. **c** Loading 1 score plot shows up regulation of R-1-Aminopropan-2-ol and ophthalmate, whereas serotonin was down regulated in class IV, CF patients. **d** Exploratory ROC was generated by PLS-DA model shows variable combinations models with

increased sensitivity (x-axis) and specificity (y-axis) and area under the curve (AUC) = 0.880. **e** R-1-Aminopropan-2-ol is up regulated in class IV in comparison to class III with an AUC = 0.906. **f** Ophthalmate is also up regulated in class IV with an AUC = 0.744. Data were normalized, transformed, and scaled by median, log, and Pareto scaling to make sure all the data are under Gaussian distribution. For paired analysis, a combination of t-test and fold change analyses are represented, where the x-axis (FDR-corrected p value), and the y-axis is true positive. Abbreviations: CF-cystic fibrosis, OPLS-DA-Orthogonal Projections to Latent Structures Discriminant Analysis

Table 6 Significantly dysregulated metabolites in Cystic Fibrosis patients between CF classes III and IV, based on Volcano Plot analysis that combines T-test and fold change analyses

Name of the metabolite	FC	log2(FC)	p-value	Log 10 (p)
R-1-Aminopropan-2-ol	0.59	− 0.756	0.000	8.11
Ophthalmate	0.54	− 0.895	0.001	3.27
Allocystathionine—Isomer	0.66	− 0.601	0.001	3.25
2,5-Dihydroxypyridine	0.65	− 0.624	0.001	2.97
Serotonin	1.88	0.911	0.003	2.56
Cystathionine—Isomer	0.66	− 0.608	0.003	2.48
Prolyl-Glutamate	1.57	0.653	0.003	2.48
Glutamyl-Tyrosine	0.65	− 0.621	0.004	2.44
Glutamyl-Glutamic acid	1.58	0.660	0.004	2.39
5-Aminopentanoic acid	1.56	0.642	0.006	2.26
Isomer of Desaminotyrosine	1.89	0.922	0.006	2.22
Gamma-Glutamylglutamic acid	1.59	0.665	0.008	2.10
3,4-Dihydroxymandelate 3-O-sulfate	0.61	− 0.705	0.023	1.64

The cut-off for these two analyses are 0.05 and 1.5, respectively

4.1.1 Dysregulated metabolites were identified between the different mutational classes.

The metabolites significantly altered among the overall CF group; classes (II–VI) demonstrated significant alteration in 20 metabolites. These identified metabolites are involved in NO, glutamine, glutamate metabolisms, and arginine biosynthesis.

The metabolomic profiling of different classes of CF identified R-1-Aminopropan-2-ol, ophthalmic acid, and glutamic acid as the top three metabolites with the highest dysregulation (Fig. 2c). R-1-Aminopropan-2-ol is amino alcohol required to synthesize cobinamides in cobamide (cobalamin cofactors) synthesis pathway. Cobamides are essential cofactors used by many enzymes, including those enzymes involved in central metabolic processes such as carbon metabolism and methionine and deoxynucleotides (Roth et al. 1996; Shelton et al. 2019). Cobinamides, act as high-affinity binders and scavengers of nitric oxide (NO) in biological systems. The action of cobinamide on NO was explored by Broderick et al. who showed that cobinamide reverses the fluid secretions stimulated by NO in *Drosophila* Malpighian tubules. The increased levels of R 1-aminopropanolol in patients with CF may indicate a mechanism through which levels of cobinamide increase and subsequently increase NO scavenging activity in these patients (Broderick et al. 2005).

We also identified ophthalmic acid, a tripeptide (L-γ-glutamyl-L-α-aminobutyrylglycine) analog of glutathione (GSH), to be significantly altered. Ophthalmic acid serves as an indicator for GSH synthesis and hepatic consumption and is a marker for oxidative stress. Besides being an anion and chloride ion channel, CFTR has been proposed to function as a transporter for other anions, including GSH. GSH within epithelial lung lining is known to reduce the

viscosity of the mucosal secretions, affects mucus hydration, and modulates the inflammatory response to infection (Wetmore et al. 2010). Ophthalmic acid was also identified in our previous work in DBS extracts in neonates with CF. It represented the pathognomonic marker of CF, serving as a proxy for assessing CFTR-mediated impaired GSH efflux (Al-Qahtani et al. 2020). In our study, we also found an increase in hypoxanthine levels, a purine nucleotide, and a product of the adenosine metabolic pathway, in the serum of CF patients compared to the healthy controls. Derivatives of xanthine have been identified as metabolites that act as activators of CFTR protein to increase the airway surface liquid volume in the lungs. An increase in our study levels may suggest a compensatory protective effect in the lungs (Quinlan et al. 1997). Our findings are contrary to Wetmore et al. who showed a decrease in the levels of both ophthalmic acid and purine nucleotides in patients with CF (Wetmore et al. 2010).

The metabolic profiling of the overall patients with CF identified an increase in symmetric dimethylarginine levels, an inactive stereoisomer of asymmetric dimethylarginine, a competitive inhibitor synthase (Reyhani et al. 2017) an enzyme responsible for the synthesis of NO. Previous studies in CF have reported abnormalities in the expression of inducible NO synthase with decreased NO production, even though concentrations of NO metabolites, nitrate, and nitrite are increased in CF airway secretions (Grasemann and Pencharz, 2013). Our findings are in line with Grasemann et al. who also demonstrated increasing levels of symmetric dimethylarginine in the airways of patients with CF and proposed that its increase could contribute to airway obstruction by reducing the synthesis of NO (Grasemann et al. 2011). The pathway analysis identified pathways relating to arginine synthesis,

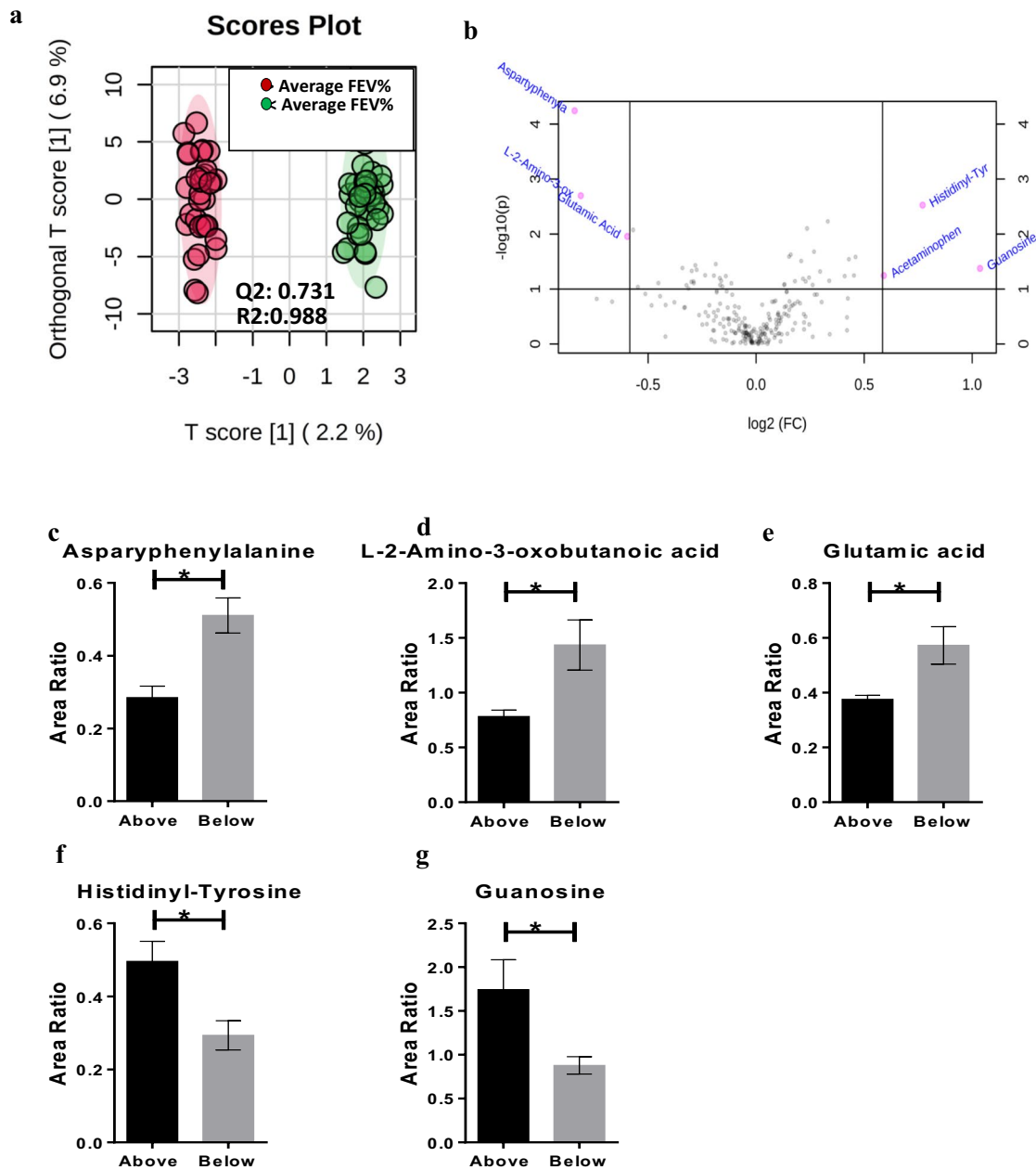


Fig. 4 Positively identified serum metabolites in CF patients above and below the average of FEV% **a** OPLS-DA plot for binary comparisons above average (red dots, $n=32$) and below average (green dots, $n=38$) with a calculated space of $R^2=0.978$ and $Q^2=0.684$ shows clear separation between the two groups. **b** Volcano plot analysis with fold change >1.5 and <0.67 . 5 metabolites were found to be significant. **c–e** Asparryphenylalanine, L-2 Amino-3-oxobutanoic acid and

Glutamic acid are down regulated in above average group. **f,g** Histidiny-Tyr and Guanosine are up regulated in the above average group. Note: that the p-values and fold changes are $\log_{10}$ and $\log_2$ transformed respectively and for fold change analyses represented in this volcano plot, the x-axis (FDR-corrected p value), and the y-axis is true positive. * Indicates significance with p-value <0.05

nitrogen metabolism, glutamine, and glutamate biosynthesis (Fig. 2d).

Independent network pathway analysis, using IPA, identified pathways related to amino acid metabolism, small molecule biochemistry, cell-to-cell signaling, and interaction and centered around regulating amino acid glutamate

(Fig. 5b). As mentioned above, glutamate is an essential metabolite that participates in numerous synthetic pathways, including the synthetic glutathione pathway. It also serves as a significant metabolic hub, as rightly depicted in the network pathway (Fig. 5b), that is involved in diverse processes besides protein synthesis, including nitrogen assimilation,

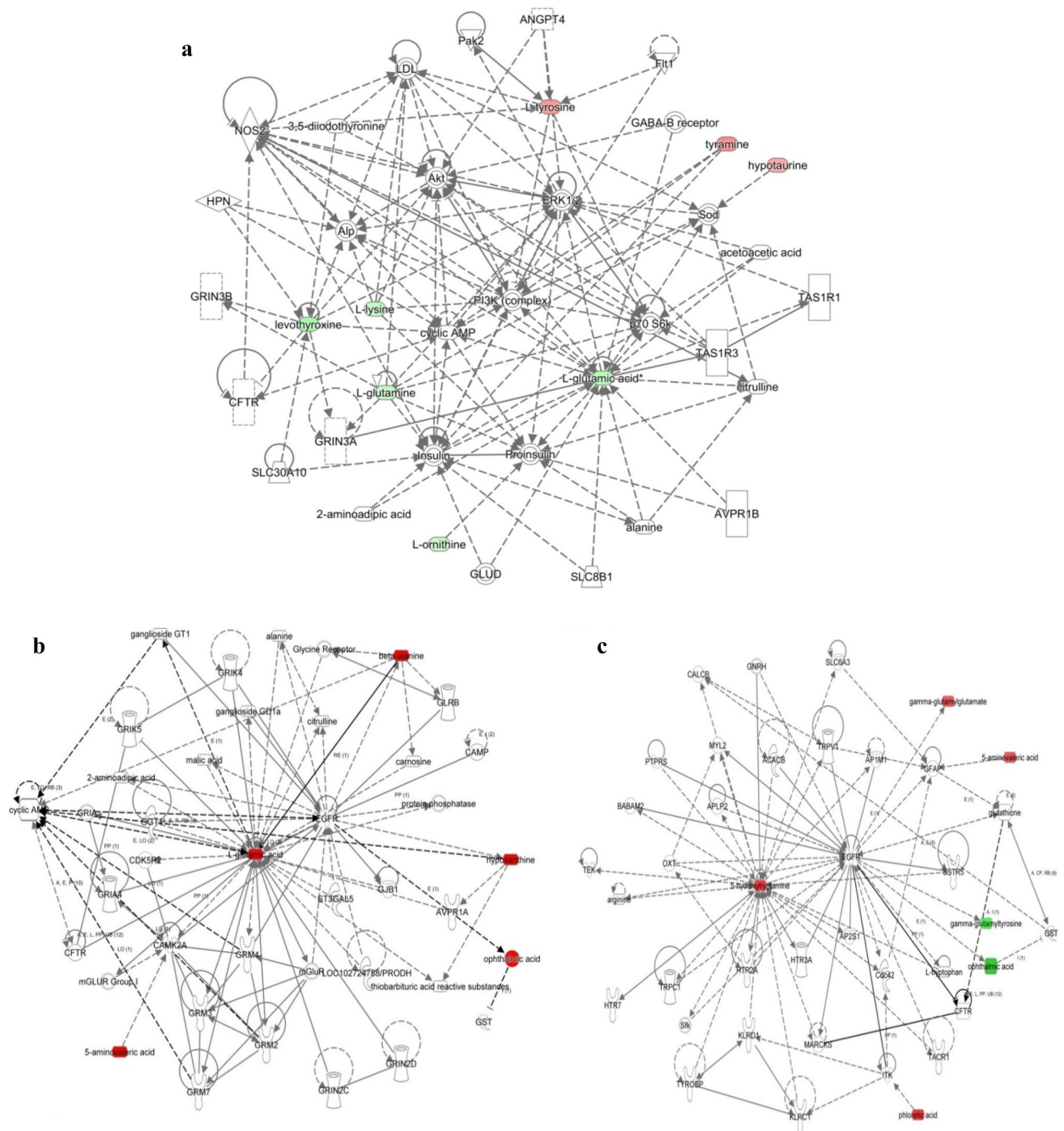


Fig. 5 Schematic representation of the highest scoring network pathways depicting the involvement of the differentially regulated metabolites **a** between patients with CF and Controls, **b** in patients with CF classes and **c** between CF classes III and IV. IPA analysis found the functional interaction network of the metabolites and proteins was related to amino acid metabolism, developmental disorder, hereditary disorder in between patients with CF and Controls, amino acid metabolism, small, molecule biochemistry, cell-to-cell signaling and

interaction in patients with CF and cell-to-cell signaling and interaction, molecular transport, small molecule biochemistry in between CF classes III and IV. Nodes in green and red correspond to down regulated and up regulated proteins, respectively. Colorless nodes were proposed by IPA and suggest potential targets functionally coordinated with the differentially abundant proteins. Solid lines indicate direct molecular interactions and dashed lines represent indirect interactions

nucleotide, amino acid, and cofactor biosynthesis and catabolism of certain amines (Walker and van der Donk 2016).

4.2 Dysregulated metabolites identified between CF class III vs class IV

A total of 13 metabolites were found to be significantly altered between functional classes; Class III and IV of CF (6 up- and 7 down-regulated) unlike the other classes, both Class III and IV demonstrate the presence of a low amount or aberrant or nonfunctional forms of the CFTR protein. The metabolites R-1-aminopropan-2-ol, ophthalmate, 3,4-dihydroxymandelate 3-o-sulfate 2,5-dihydropyridine, glutamyl-tyrosine, cystathionine, and allocystathionine levels were decreased in class III vs. class IV. The main canonical pathway identified with altered regulation was identified to be cystathionine and homocysteine degradation pathways.

The metabolite (R)-1-Aminopropan-2-ol, identified in the CF group, was also significantly decreased in class III vs. class IV, indicating a reduced NO scavenging activity and cobinamide synthesis in CF class III wherein there is a gating defect in the CFTR protein. The dipeptides glutamyl-glutamic acid, gamma-glutamylglutamic acid, prolyl glutamate, and serotonin were increased in class III vs. class IV, indicating that there is a recycling of glutamate through the formation of dipeptides. This salvage pathway seems to be enhanced to overcome the conductance defect in the CFTR protein, which generally conducts glutathione besides the anions. The salvage pathway's involvement was also confirmed by demonstrating decreased enzyme glutamyl transferase levels in our independent serum proteomics study on the same patients (unpublished data). The reduced activity of this enzyme may reflect the heightened redox and oxidative state caused by CF that also concurrently leads to altered levels of glutamate, glutamylglutamic acid, as seen in the present metabolomic profile of this study cohort along with others including mercapturic acid and cystine that are intermediates in the synthesis of glutathione via the glutamyl cycle. An increase in the dipeptide glutamylglutamate was observed in this study, which is known to regulate multiple pathways, including glutathione synthesis and glutathione-mediated detoxification pathways. The role of glutathione in CF is well documented in the literature. Both the tripeptide and its metabolites, including ophthalmic acid, promote CFTR cell-surface expression and channel activation in CF epithelial cells. The beneficial effects of glutathione have prompted its use as a treatment modality in patients with CF to decrease mucosal secretions' viscosity.

Moreover, CFTR plays an essential role in GSH uptake from the diet and transport processes in the lung (Kariya et al. 2007; Wetmore et al. 2010). The levels of glutamyl glutamic acid were increased while ophthalmic acid was decreased in class III vs. class IV patients. A decrease in

ophthalmic acid levels may point to increased oxidative stress in CF class III compared to class IV. An increase in the dipeptides glutamyl-glutamic acid, glutamylglutamate, prolyl glutamate in class III vs. class IV, suggests an intracellular existence extracellular pool of dipeptide intermediates involved in glutamate recycling. Recycling through these dipeptides helps overcome the conductance defect for glutathione in the CFTR protein in patients with CF, such that its intracellular and extracellular levels are maintained.

The differentially regulated canonical pathways identified in our study were cystathionine and homocysteine degradation pathways that result in the formation of ammonia. L- cystathionine levels were decreased between class III and class IV, indicating that cystathionine degradation was reduced in the class III group (Table 6). Another interesting finding in this group was the decrease in serotonin levels (5-hydroxytryptamine), a neurotransmitter and a hormone synthesized from the amino acid tryptophan, levels between CF Class III and IV (Table 6). In a recent study using mouse models, serotonin was found to aggravate pulmonary fibrosis by promoting inflammation and oxidative stress and activating genes associated with fibrosis (Zhang et al. 2018). An increase in serotonin's circulating blood levels, first reported by Partington et al. was associated with a decrease in the FEV1% in children with CF without changes in its excretory metabolite (Partington and Ferguson 1977). Measuring serotonin can be proposed to differentiate between CF class III and IV and be used as a therapeutic target. The biological implications of the altered metabolites between CF class III and IV and their impact on various cellular pathways were also examined through network pathway analysis. Pathway analyses identified the pathways relating to cell-to-cell signaling and interaction, molecular transport, small molecule biochemistry as significantly altered between the classes and focussed around the deregulation of serotonin and EGFR (Fig. 5c). Focusing on these pathways and the metabolites in future studies can help better diagnose or as pharmacological therapy targets.

4.3 Metabolites altered with a Lung function of CF patients

We investigated the correlation between patients' serum metabolome and their lung function in the present study, measured by spirometry tests as the predicted FEV1%. FEV1% is used to diagnose and assess obstructive lung diseases and is an established CF progression marker, with decreased FEV1% values often related to increased risk of death. The binary comparison was carried out to compare the metabolites altered in patients with an FEV1% above and below the average values. Significant changes

in amino acid aspartyl phenylalanine levels, L-2 amino-3-oxobutanoic acid, and Glutamic acid (Fig. 4c-e) were found to be decreased. In contrast, histidiny-tyrosine and guanosine (Fig. 4f-g) were increased in the above-average FEV1% group compared to the below-average FEV1% group. These amino acids' concerted actions may play a role in increasing the levels of guanosine, a purine nucleoside. Schwiebert et al. showed that heterotrimeric GTP-binding proteins restore cAMP-activated Chloride currents in airway epithelial cells isolated from CF patients (Schwiebert et al. 1992). Elevations in guanosine, which is the crucial metabolite in the formation of GTP-binding proteins, might be a compensatory mechanism to restore the chloride anion activity.

5 Conclusion

We used a chemical isotope-labeled mass spectrometry-based metabolomics approach to create an untargeted metabolomics profile for CF in young adult patients. In addition, we have also identified for the first time the different metabolites altered among the CF mutational classes (II-VI) and between the class III and IV. The differentially expressed metabolites related to diverse clinically relevant molecular pathways regulating glutathione and arginine synthesis and metabolism of amino acids, namely, glutamine, glutamate, and arginine metabolisms, with a high significance. Two metabolites, 3,4-dihydroxymandelate-3-O-sulfate, and 5-Aminopentanoic acid, were common between the three analyses and may represent highly sensitive CF biomarkers. Although CF is considered a single disease, our study shows that genotype and mutations alter the metabolisms differently. Therefore, better health outcomes necessitate personalizing patient health outcomes based on the metabolites' alterations with the genotype.

Acknowledgements The authors would like to express their gratitude to the Executive Director of the Research Centre, King Faisal Specialist Hospital and Research Centre, to Dr. Brian Meyer, Chairman of the Department of Genetics, for the funding supports, and to all the patients who contributed to this project. The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for funding this research through the Research Group Project no. RGP-334. The work performed at the Li-node of the Metabolomics Innovation Centre (TMIC) of Canada was partially funded by Genome Canada and Canada Foundation for Innovation.

Author contributions: AM analyzed the data and wrote the first draft of the manuscript. MJ compiled the figures and tables and contributed to drafting the manuscript. LL and XG conducted the metabolomics experiment, IN and MAJ recruited patients and provided their clinical data and samples. HB performed the proteomics analysis. MD critically revised the manuscript, and AMAR designed the study, supervised

experiments, data analysis, and finalized the manuscript. All authors approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors have no potential conflict of interest.

Informed consent The authors declare that all the patients who participated in this study provided informed consent to use their biological material.

References

- Al-Qahtani, W., Abdel Jabar, M., Masoos, A., Jacob, M., Nizami, I., Dasouki, M., & Abdel Rahman, A. M. (2020). Dried blood spot-based metabolomic profiling in adults with cystic fibrosis. *Journal of Proteome Research*, 19, 2346–2357.
- Benabdelkamel, H., Alamri, H., Okla, M., Masood, A., Abdel Jabar, M., Alanazi, I. O., et al. (2020). Serum-based proteomics profiling in adult patients with cystic fibrosis. *International Journal of Molecular Science*, 21, 7415.
- Bolourian, A., & Mojtahedi, Z. (2018). Streptomyces, shared microbiome member of soil and gut, as 'old friends' against colon cancer. *FEMS Microbiology Ecology*. <https://doi.org/10.1093/femsec/fiy120>.
- Boto-Ordóñez, M., Urpi-Sarda, M., Queipo-Ortuno, M. I., Tulipani, S., Tinahones, F. J., & Andres-Lacueva, C. (2014). High levels of Bifidobacteria are associated with increased levels of anthocyanin microbial metabolites: a randomized clinical trial. *Food Function*, 5, 1932–1938.
- Broderick, K. E., Singh, V., Zhuang, S., Kambo, A., Chen, J. C., Sharma, V. S., et al. (2005). Nitric oxide scavenging by the cobalamin precursor cobinamide. *Journal of Biological Chemistry*, 280, 8678–8685.
- Callery, P. S., & Geelhaar, L. A. (1984). Biosynthesis of 5-aminopentanoic acid and 2-piperidone from cadaverine and 1-piperidine in mouse. *Journal of Neurochemistry*, 43, 1631–1634.
- Callery, P. S., & Geelhaar, L. A. (1985). 1-Piperidine as an in vivo precursor of the gamma-aminobutyric acid homologue 5-aminopentanoic acid. *Journal of Neurochemistry*, 45, 946–948.
- Calvano, S. E., Xiao, W., Richards, D. R., Felciano, R. M., Baker, H. V., Cho, R. J., et al. (2005). A network-based analysis of systemic inflammation in humans. *Nature*, 437, 1032–1037.
- Castellani, C., & Assael, B. M. (2017). Cystic fibrosis: A clinical view. *Cellular and Molecular Life Sciences*, 74, 129–140.
- Castellani, C., Cuppens, H., Macek, M., Jr., Cassiman, J. J., Kerem, E., Durie, P., et al. (2008). Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice. *Journal of Cystic Fibrosis*, 7, 179–196.
- Chen, D., Su, X., Wang, N., Li, Y., Yin, H., Li, L., & Li, L. (2017). Chemical isotope labeling LC-MS for monitoring disease progression and treatment in animal models: plasma metabolomics study of osteoarthritis rat model. *Scientific Reports*, 7, 40543.
- Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., et al. (2018). MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Research*, 46, W486–W494.
- Dahabiyeh, L. A. Malkawi, A. K. Wang, X. Colak, D. Mujamammi, A. H. Sabi, et al. (2020). Dexamethasone-induced perturbations in tissue metabolomics revealed by chemical isotope labeling LC-MS analysis. *Metabolites*, 10, 42.

- Davis, P. B., Drumm, M., & Konstan, M. W. (1996). Cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, 154, 1229–1256.
- de Boeck, K., Zolin, A., Cuppens, H., Olesen, H. V., & Viviani, L. (2014). The relative frequency of CFTR mutation classes in European patients with cystic fibrosis. *Journal of Cystic Fibrosis*, 13, 403–409.
- Eisenhofer, G., Aneman, A., Hooper, D., Rundqvist, B., & Friberg, P. (1996). Mesenteric organ production, hepatic metabolism, and renal elimination of norepinephrine and its metabolites in humans. *Journal of Neurochemistry*, 66, 1565–1573.
- Esther, C. R. JR. Turkovic, L. Rosenow, T. Muhlebach, M. S. Boucher, R. C. Ranganathan, S. & Stick, S. M. (2016). Metabolic biomarkers predictive of early structural lung disease in cystic fibrosis. *European Respiratory Journal*, 48, 1612–1621.
- Forester, S. C., & Waterhouse, A. L. (2008). Identification of Cabernet Sauvignon anthocyanin gut microflora metabolites. *Journal of Agriculture and Food Chemistry*, 56, 9299–9304.
- Gadsby, D. C., Vergani, P., & Csanady, L. (2006). The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature*, 440, 477–483.
- Galant, S. P., Norton, L., Herbst, J., & Wood, C. (1981). Impaired beta adrenergic receptor binding and function in cystic fibrosis neutrophils. *Journal of Clinical Investigation*, 68, 253–258.
- Grasemann, H., Al-Saleh, S., Scott, J. A., Shehnaz, D., Mehl, A., Amin, R., et al. (2011). Asymmetric dimethylarginine contributes to airway nitric oxide deficiency in patients with cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, 183, 1363–1368.
- Grasemann, H., & Pencharz, P. B. (2013). Arginine metabolism in patients with cystic fibrosis. *Journal of Pediatrics*, 163, 317–319.
- Grasemann, H., Schwiertz, R., Grasemann, C., Vester, U., Racke, K., & Ratjen, F. (2006). Decreased systemic bioavailability of L-arginine in patients with cystic fibrosis. *Respiratory Research*, 7, 87.
- Guo, K., & Li, L. (2009). Differential 12C-/13C-isotope dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome. *Analytical Chemistry*, 81, 3919–3932.
- Huan, T., & Li, L. (2015). Counting missing values in a metabolite-intensity data set for measuring the analytical performance of a metabolomics platform. *Analytical Chemistry*, 87, 1306–1313.
- Huan, T., Wu, Y., Tang, C., Lin, G., & Li, L. (2015). DnsID in MyCompoundID for rapid identification of dansylated amine- and phenol-containing metabolites in LC-MS-based metabolomics. *Analytical Chemistry*, 87, 9838–9845.
- Jacob, M., Gu, X., Luo, X., Al-Mousa, H., Arnaout, R., Al-Saud, B., et al. (2019). Metabolomics distinguishes DOCK8 deficiency from atopic dermatitis: towards a biomarker discovery. *Metabolites*, 9, 274.
- Kariya, C., Leitner, H., Min, E., van Heeckeren, C., van Heeckeren, A., & Day, B. J. (2007). A role for CFTR in the elevation of glutathione levels in the lung by oral glutathione administration. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 292, L1590–L1597.
- Laguna, T. A., Reilly, C. S., Williams, C. B., Welchlin, C., & Wendt, C. H. (2015). Metabolomics analysis identifies novel plasma biomarkers of cystic fibrosis pulmonary exacerbation. *Pediatric Pulmonology*, 50, 869–877.
- Li, C., Ramjeesingh, M., Wang, W., Garami, E., Hewryk, M., Lee, D., et al. (1996). ATPase activity of the cystic fibrosis transmembrane conductance regulator. *Journal of Biological Chemistry*, 271, 28463–28468.
- Li, L., Li, R., Zhou, J., Zuniga, A., Stanislaus, A. E., Wu, Y., et al. (2013). MyCompoundID: using an evidence-based metabolome library for metabolite identification. *Analytical Chemistry*, 85, 3401–3408.
- Long, F., Liu, H., Hahn, C., Sumazin, P., Zhang, M. Q., & Zilberstein, A. (2004). Genome-wide prediction and analysis of function-specific transcription factor binding sites. *Silico Biology*, 4, 395–410.
- Maiuri, L., Raia, V., & Kroemer, G. (2017). Strategies for the etiological therapy of cystic fibrosis. *Cell Death and Differentiation*, 24, 1825–1844.
- Makni, M., Chtourou, Y., Barkallah, M., & Fetoui, H. (2012). Protective effect of vanillin against carbon tetrachloride (CCl₄)-induced oxidative brain injury in rats. *Toxicology and Industrial Health*, 28, 655–662.
- Marson, F. A. L., Bertuzzo, C. S., & Ribeiro, J. D. (2016). Classification of CFTR mutation classes. *Lancet Respiratory Medicine*, 4, e37–e38.
- Moran, A., Milla, C., Ducret, R., & Nair, K. S. (2001). Protein metabolism in clinically stable adult cystic fibrosis patients with abnormal glucose tolerance. *Diabetes*, 50, 1336–1343.
- Muhlebach, M. S., Sha, W., Macintosh, B., Kelley, T. J., & Muenzer, J. (2019). Metabonomics reveals altered metabolites related to inflammation and energy utilization at recovery of cystic fibrosis lung exacerbation. *Metabolism Open*, 3, 100010.
- Ogawara, H. (2019). Comparison of antibiotic resistance mechanisms in antibiotic-producing and pathogenic bacteria. *Molecules*, 24.
- Painter, R. G., Valentine, V. G., Lanson, N. A., Jr., Leidal, K., Zhang, Q., Lombard, G., et al. (2006). CFTR Expression in human neutrophils and the phagolysosomal chlorination defect in cystic fibrosis. *Biochemistry*, 45, 10260–10269.
- Partington, M. W., & Ferguson, A. C. (1977). Serotonin metabolism in cystic fibrosis. *Archives of Disease in Childhood*, 52, 386–390.
- Quinlan, G. J., Lamb, N. J., Tilley, R., Evans, T. W., & Gutteridge, J. M. (1997). Plasma hypoxanthine levels in ARDS: implications for oxidative stress, morbidity, and mortality. *American Journal of Respiratory and Critical Care Medicine*, 155, 479–484.
- Ratjen, F., Bell, S. C., Rowe, S. M., Goss, C. H., Quittner, A. L., & Bush, A. (2015). Cystic fibrosis. *Nature Review Disease Primers*, 1, 15010.
- Reyhani, A., Celik, Y., Karadag, H., Gunduz, O., Asil, T., & Sut, N. (2017). High asymmetric dimethylarginine, symmetric dimethylarginine and L-arginine levels in migraine patients. *Neurology Science*, 38, 1287–1291.
- Rogan, M. P., Stoltz, D. A., & Hornick, D. B. (2011). Cystic fibrosis transmembrane conductance regulator intracellular processing, trafficking, and opportunities for mutation-specific treatment. *Chest*, 139, 1480–1490.
- Roth, J. R., Lawrence, J. G., & Bobik, T. A. (1996). Cobalamin (coenzyme B12): synthesis and biological significance. *Annual Review of Microbiology*, 50, 137–181.
- Schneider, E., & Hunke, S. (1998). ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiology Reviews*, 22, 1–20.
- Schwiebert, E. M., Kizer, N., Gruenert, D. C., & Stanton, B. A. (1992). GTP-binding proteins inhibit cAMP activation of chloride channels in cystic fibrosis airway epithelial cells. *Proceedings of the National Academy of Sciences USA*, 89, 10623–10627.
- Shelton, A. N., Seth, E. C., Mok, K. C., Han, A. W., Jackson, S. N., Haft, D. R., & Taga, M. E. (2019). Uneven distribution of cobamide biosynthesis and dependence in bacteria predicted by comparative genomics. *ISME Journal*, 13, 789–804.
- Stefely, J. A., & Pagliarini, D. J. (2017). Biochemistry of mitochondrial coenzyme Q biosynthesis. *Trends in Biochemical Sciences*, 42, 824–843.
- Walker, M. C., & van der Donk, W. A. (2016). The many roles of glutamate in metabolism. *Journal of Industrial Microbiology and Biotechnology*, 43, 419–430.
- Wetmore, D. R., Joseloff, E., Pilewski, J., Lee, D. P., Lawton, K. A., Mitchell, M. W., et al. (2010). Metabolomic profiling reveals biochemical pathways and biomarkers associated with pathogenesis

- in cystic fibrosis cells. *Journal of Biological Chemistry*, 285, 30516–30522.
- Winter-De, D. E., Groot, K. M., & Van Der Ent, C. K. (2005). Nitric oxide in cystic fibrosis. *Journal of Cystic Fibrosis*, 4(Suppl 2), 25–29.
- Wolak, J. E., Esther, C. R., Jr., & O'Connell, T. M. (2009). Metabolomic analysis of bronchoalveolar lavage fluid from cystic fibrosis patients. *Biomarkers*, 14, 55–60.
- Wu, Y., & Li, L. (2012). Determination of total concentration of chemically labeled metabolites as a means of metabolome sample normalization and sample loading optimization in mass spectrometry-based metabolomics. *Analytical Chemistry*, 84, 10723–10731.
- Zardini Buzatto, A., Abdel Jabar, M., Nizami, I., Dasouki, M., Li, L., & Abdel Rahman, A. M. (2020). Lipidome alterations induced by cystic fibrosis, CFTR mutation, and lung function. *Journal of Proteome Research*. <https://doi.org/10.1021/acs.jproteome.0c00556>.
- Zhang, J., Cui, R., Feng, Y., Gao, W., Bi, J., Li, Z., & Liu, C. (2018). Serotonin exhibits accelerated bleomycin-induced pulmonary fibrosis through TPH1 knockout mouse experiments. *Mediators of Inflammation*, 2018, 7967868.
- Zhou, R., Tseng, C. L., Huan, T., & Li, L. (2014). IsoMS: automated processing of LC-MS data generated by a chemical isotope labeling metabolomics platform. *Analytical Chemistry*, 86, 4675–4679.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.