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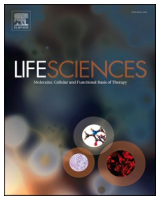

Characterizing COPD phenotypes with a targeted signaling lipids metabolomics approach

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ABSTRACT

Aims: This study aimed to elucidate clinically-relevant classifications of COPD using a targeted metabolomics approach focusing on signaling lipids.
Materials and methods: Using a targeted LC-MS/MS platform, 166 metabolites including free fatty acids, prostaglandins, isoprostanes, lysophospholipids, endocannabinoids, and bile acids were profiled in a cohort of 49 COPD patients. The study integrated metabolomic data with clinical parameters to identify key metabolites and related pathways for various COPD classification systems including Global Initiative for Chronic Obstructive Lung Disease (GOLD) grading stages, Koninklijk Nederlands Genootschap voor Fysiotherapie (KNGF, Royal Dutch Society for Physiotherapy) profiles, and Systemic (SYS) subtypes and explored the association of these classification systems.
Key findings: The GOLD stages showed correlations with 15 metabolites, including lysophospholipids, oxylipins, and bile acids. KNGF profiles were linked to 13 metabolites, predominantly lysophospholipids, while SYS subtypes were associated with 9 metabolites, mainly oxylipins. A specific cluster of oxylipins, including HETEs and HDoHEs, was notably correlated to prognostic factors of COPD.
Significance: This study identified distinct metabolic patterns associated with GOLD stages, KNGF profiles, and SYS subtypes. Additionally, the findings indicate that 14-HDoHE/DHA may serve as a potential biomarker for COPD exacerbation and suggest possible therapeutic targets for COPD, including pathways involving lipoxygenases, G-protein coupled receptors, and the Farnesoid X receptor.

Abbreviations: COPD, chronic obstructive pulmonary disease; GOLD, global initiative for chronic obstructive lung disease; FEV1, forced expiratory volume in the first 1 s; FVC, forced vital capacity; KNGF, Koninklijk Nederlands Genootschap voor Fysiotherapie (Royal Dutch Society for Physiotherapy); CCQ, clinical COPD questionnaire; CAT, COPD assessment test; SYS, systemic; mMRC, modified medical research council dyspnea scale; MSQ, Marshall questionnaire; MUST, malnutrition universal screening tool; SARC-F, strength, assistance walking, rising from a chair, climbing stairs, and falls; 6MWT, six-minute walk test; MISS, measure it super simple; EDTA, ethylenediaminetetraacetic acid; UHPLC-MS/MS, ultra-high performance liquid chromatography–mass spectrometry; BMI, body mass index; GPRs, G-protein coupled receptors; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; RhoA, Ras homolog gene family member A; ROCK, Rho-associated coiled-coil-containing kinase; ALI, acute lung injury; CCR3, C-C chemokine receptor type 3; IL-17, interlukin-17; LOX, lipoxygenase; ROC, receiver-operating characteristic; AUC, area under curve; COX, cyclooxygenase; CYPs, cytochrome P450; COVID-19, coronavirus disease 2019; ARDS, acute respiratory distress syndrome; FXR, farnesoid X receptor; NF-κB, Nuclear factor kappa B.

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1. Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a heterogeneous lung disease caused by abnormalities of the airways and/or alveoli, resulting in dysfunction of airflow obstruction and chronic respiratory symptoms such as cough, sputum production, dyspnea and exacerbation [1]. COPD is also the third leading cause of death worldwide [2,3]. The estimated global prevalence of COPD among individuals aged 30–79 was approximately 391.9 million in 2019 [4]. One of the main difficulties in treating COPD is disease heterogeneity. Diverse risk factors including tobacco smoking, inhalation of pollutants, age, physical condition, comorbidities and early-life events all contribute to the observed heterogeneity of COPD demonstrating the need for a more personalized treatment [5,6]. However, current treatment is mainly based on the severity of airflow obstruction, neglecting the distinctions in etiology and pathology among COPD patients [1]. Consequently, targeted therapeutic approaches to treat COPD are still limited even though multiple pharmacological and non-pharmacological treatments are available. Moreover, pharmacological treatments are mainly aimed at symptom relief and, in many cases, continuous adjustments in medication decisions take place after the initial therapy. On the other hand, non-pharmacological treatments are aimed at pulmonary rehabilitation, improving nutrition and physiological condition [1,7,8].

The medical treatment of COPD is based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) grading system in which patients are categorized into 4 grades (GOLD 1–4), depending on airflow limitation severity, characterized by the ratio of the forced expiratory volume and the forced vital capacity (FEV1/FVC ratio) [1]. Other factors also guide COPD treatment including additional symptoms such as dyspnea, previous history of exacerbations and comorbidities. Despite medical treatment by the general practitioner, an impaired physical, emotional and/or social functioning has been frequently reported in Dutch primary care patients with chronic obstructive pulmonary disease (COPD). These abnormalities can co-occur in different combinations, regardless of the degree of airflow limitation [9–11]. Disease stability, disease burden, physical capacity and physical activity are important traits to get the right patient allocated to the right type of exercise-related care and at the right moment, irrespective of the degree of airflow limitation [12].

In the Netherlands, the Koninklijk Nederlands Genootschap voor Fysiotherapie (KNGF, Royal Dutch Society for Physiotherapy) has developed a physiotherapeutic diagnosis and treatment guideline for COPD patients based on symptoms, physical activity and physical capacity [12]. In this guideline, patients are classified into one of 6 profiles depending on the presence of exacerbations and the outcome of the Clinical COPD Questionnaire (CCQ) or COPD Assessment Test (CAT) as well as the results of the 6-minute walk test and step count measurements using an activity tracker. Initial treatment can differ based on the KNGF profile of the patient: Patients in profile 1 and profile 2 are recommended to participate in regular fitness and sports activities, physiotherapy is offered to patients with profiles 3 to 5. For patients in profile 6, the guideline recommends starting with third-line screening, followed by third-line pulmonary rehabilitation if necessary, and then proceeding with first-line treatment [12].

To account for the complexity and heterogeneity of COPD and to personalize treatment, several phenotypes of COPD have been identified that seem to be associated with clinical outcomes [13–17]. A COPD phenotype can be defined as a unique set of measurable characteristics such as cough, wheeze, shortness of breath and chest tightness. The classification and identification of several COPD phenotypes have evolved from the 3 traditional subgroups of COPD known as chronic bronchitis, emphysema and asthma to more specific and well-defined phenotypes [13]. The attention to COPD subgroups has also shifted from solely pathological manifestations to the combination of symptoms and etiology of COPD. Nevertheless, it is still a challenge to transfer these phenotypes to personalized therapeutic approaches in routine care, since we first need a better understanding of the pathophysiological mechanisms behind these phenotypes [14,18,19].

Simultaneously, several COPD phenotypes have been described and applied to clinical practice in China for personalized treatment [20]. There are mainly 4 systemic (SYS) phenotypes for stable COPD which are formed through a comprehensive analysis of clinical symptoms and signs [21]. The SYS phenotype is characterized by disease cause, location, nature and tendency, not just the depiction of symptoms. Therefore, a patient-tailored treatment program could be developed based on patients' specific SYS phenotype [22]. Nevertheless, there are few studies on elucidating underlying biological mechanisms of these different COPD classification systems.

Recently, it has been shown that metabolic disturbances are closely related to several key pathological processes of COPD [23–25]. Specific lipid metabolites contributing to the inflammation response seem to play a role in diagnosis, progression and prognosis of COPD [24]. For instance, variations of lysoglycerophospholipids, phosphatidylglycerols and glycerol lipids have been found to play a role in the shift from the stable to the acute stage (exacerbation) of COPD. In addition, it has been shown that plasmalogen relates to peroxisomal dysfunction and oxidative stress [26,27]. Metabolomics research for COPD has predominantly focused on inspecting metabolite changes and distinguishing traditional COPD phenotypes, such as emphysema and asthma [25,26]. However, most of these studies have employed untargeted metabolomics, resulting in lower specificity and reliability of targets [28–31].

The aim of this study is to characterize and compare three COPD classification systems (GOLD, KNGF, SYS) using targeted metabolomics measurements in plasma samples which might lead to new options for personalized treatment of COPD patients. Therefore, to profile targets that are especially relevant to COPD and obtain reliable metabolomics data, this study utilized a targeted metabolomics platform concentrating on metabolites primarily involved in inflammation, a crucial pathway in the mechanism of COPD. This LC-MS/MS based method covers 261 metabolites including free fatty acids, prostaglandins, isoprostanes, lysophospholipids, endocannabinoids and bile acids [32], and has been applied to various diseases or pathophysiological conditions [33–35]. In addition, by integrating these metabolomics data with clinical parameters, we aim to reveal distinct metabolic patterns across GOLD stages, KNGF profiles and SYS phenotypes. This explorative study will provide insights and evidence in connecting metabolic disturbance and different COPD classification systems, rather than developing a predictive model or biomarker to identify COPD stages.

2. Material and methods

2.1. Subjects and study design

In this cross-sectional research, patient recruitment was conducted by Zuyd University of Applied Sciences and the Zuyderland Medical Center between 2021 and 2022. Patients were recruited from private physiotherapy practices and the Zuyderland Medical Center, with informed consent obtained for inclusion in the study. The study was approved in 5th October 2020 by the medical ethical committee Brabant, the Netherlands (NL74360.028.20/P2051) and was conducted in accordance with the latest revised ethical guidelines of the Declaration of Helsinki. Adult participants with a medical diagnosis of COPD and age above 18 years were included. Patients with an acute exacerbation were excluded. The following baseline characteristics were collected: age, gender, BMI and COPD-related parameters such as exacerbation history, smoking history and GOLD-stage [1]. Smoking history was determined solely by whether they had smoked or not, without consideration of duration.

2.2. Clinical measurements

The following questionnaires were used: the modified Medical

Research Council dyspnea scale (mMRC) [36], Clinical COPD Questionnaire (CCQ) [37], short exacerbation questionnaire [38], physical activity questionnaire (Marshall questionnaire, MSQ) [39], malnutrition universal screening tool (MUST) and self-screening tool for sarcopenia (SARC-F) [40]. The physical capacity of participants was assessed by a six-minute walk test (6MWT) [41]. The predicted six-minute walk distance was calculated based on gender, age and BMI [42]. The physical activity was measured by an activity tracker, the Measure It Super Simple activity tracker (MISS) since this tracker can measure several activities of daily living validly and is user-friendly for an elderly population [43]. The KNGF profile of each patient was determined according to the presence of exacerbation-related hospital admission, the degree of disease burden and physical activity and capacity results [12].

In addition, participants who were classified under profile 6 should go for a pre-pulmonary rehabilitation assessment to determine the appropriate treatment allocation. Since treatment allocation was not relevant to the research question, patients under profile 6 in this study were assigned to the profile following the pre-pulmonary rehabilitation assessment. This approach allowed for the inclusion of patients across a wide range of clinical complexities, from low to high.

SYS phenotypes were assessed using a systemic symptoms questionnaire. This questionnaire is based on symptoms that are associated with COPD according to Traditional Chinese Medicine (TCM) theory [44]. The symptom questionnaire can be used to assess symptoms related to the following 4 TCM phenotypes: Lung Qi Deficiency, Lung Qi and Spleen Qi Deficiency, Lung Qi and Spleen Qi and Kidney Yang deficiency, and Lung Qi and Spleen Qi and Kidney Yang and Kidney Yin Deficiency. An algorithm was used to calculate the SYS phenotype scores (Table S3 and Supplementary materials). For analysis, SYS phenotype 3 and 4 were merged into phenotype 3 because of the small sample sizes in both groups and their similar characteristics when compared to phenotype 1 and 2.

2.3. Sample collection and metabolic profiling

A fasting EDTA plasma sample of 500 μL was collected from each of the patients and stored at −80 °C until analysis. The plasma samples were measured at the Metabolomics and Analytical Centre of Leiden Academic Centre for Drug Research (Leiden University) with a comprehensive UHPLC-MS/MS method targeting signaling lipids [32]. This platform covers a total of 261 targets including free fatty acids (omega-3, omega-6, omega-9), oxylipins (isoprostanes, prostaglandins and other oxidized lipids), lysophospholipids, sphingosine lipids, endocannabinoids and bile acids.

2.4. Data pre-processing and quality control

Sciex OS (AB SCIEX, Version 2.1.6) was used to integrate metabolite peaks from raw LC-MS/MS data. The relative concentration was calculated from the area of targets divided by the area of assigned internal standard. The calibration lines were made by both neat and plasma samples. An in-house data quality control was performed to discriminate reportable targets with not detected or less confident targets, the relative standard deviation (RSD) of Quality Control (QC) samples, composed by pooled study samples, and background signal, based on QC samples and blank samples, were inspected. The targets with RSD of QC above 30 % or background signal over 40 % were excluded from statistical analyses. The targets with missing values in >20 % of samples were also excluded. The QC samples and targets were plotted after exclusion (Figs. S3 and S4). The correction using QC samples will be applied to address batch effects in cases involving 2 or more batches, although this study only included 1 batch. A total of 139 metabolites passed the criteria, which are listed in Table S1. Moreover, the sums of metabolites within the same lipid subclass and ratios of specific targets to their precursors were also calculated to provide detailed information for metabolic profiling (Table S2). Consequently, 139 detectable lipids, 5 lipid subclass sums and 22 lipid ratios amounted to 166 variables used for subsequent statistical analyses.

2.5. Statistical analyses

Prior to the analysis, the relative concentrations of all signaling lipids were $\log_2$-transformed and scaled to achieve a normal distribution and comparability among the targets. The missing values were replaced by one-tenth of the minimum value observed from the respective metabolite. Furthermore, to eliminate the interference from possible confounders, gender, age and Body Mass Index (BMI) were inspected with an independent *t*-test and Pearson correlation analysis.

To explore the underlying metabolic disturbances of COPD classifications, 3 analytical methods were performed independently for better understanding. Ordinal regression, designed for predicting ordinal variables, was utilized in this study to identify targets potentially driving COPD classifications. Only S1targets with $p \leq 0.05$ were selected for analysis and visualization using effects plots [45] which indicates how the probability distribution varies across different classifications with changes in metabolite abundance. In the meantime, Analysis of Covariance (ANCOVA), aiming to compare variances across different groups, was performed to discern metabolites that vary across COPD classifications. The *p* values were produced by one-way ANCOVA for multi groups and Benjamini–Hochberg [46] for pairwise comparisons. The abundance of metabolites was expressed by estimated marginal (EM) means with high and low end of confidence intervals after correction for age and BMI [47]. The effect size was evaluated by partial eta squared (η_p^2), with criteria for small = 0.01; medium = 0.06; large = 0.14. In addition, partial Spearman correlation analysis, appropriate for assessing the strength of association between two variables, was conducted to examine the correlations among clinical characteristics, metabolites, and COPD categories. The targets with $p \leq 0.05$ were highlighted with their corresponding correlation coefficients. Moreover, additional criteria were applied, requiring absolute value of correlation coefficients >0.35 [48] to achieve at least a moderate correlation. All the statistical analyses in this research were performed using R (version 4.3.2) along with following R packages: “MASS” and “effects” for ordinal regression, “rstatix” and “emmeans” for ANCOVA, “corrplot” and “ppcor” for Spearman correlation analysis.

3. Results

3.1. Baseline characteristics of participants

A total of 56 subjects were included in the study. Blood samples were available from 49 subjects, 18 male and 31 female. Data from these 49 subjects were used for the subsequent analyses. The descriptive characteristics and category information of study participants are shown in Table 1. The classification of the patients according to GOLD stage, KNGF profile and SYS phenotype are presented in Fig. 1. There were 2 missing values in GOLD stages and one missing value in KNGF profiles. Subjects with an unknown GOLD stage or KNGF profile were excluded from the corresponding analyses. There were no patients in GOLD stage 1 and KNGF profile 3. 35 participants who were initially classified under KNGF profile 6 were reassigned to other KNGF profiles:12 to profile 2, 18 to profile 4, and 5 to profile 5. No correlations were observed between GOLD stages, KNGF profiles and SYS phenotypes, as correlation coefficients between GOLD and KNGF, GOLD and SYS, KNGF and SYS were 0.03, 0.02 and 0.18 respectively.

In addition, gender, age and BMI were identified as confounders for certain metabolites (Table S4 and Fig. S1). 8 targets showed obvious gender bias and were therefore excluded from the results. Additionally, age was associated with a cluster of lysophospholipids while BMI was linked to fatty acids and oxylipins. Consequently, both age and BMI were taken into mathematical models as covariates.

Table 1
Participant characteristics.

Characteristic	Total cohort	Male	Female
Number of subjects	49	18	31
Age (years)	68.3 ± 8.3	70.8 ± 7.4	66.8 ± 8.5
Weight (kg)	76.2 ± 16.2	78.6 ± 16.2	74.8 ± 16.3
BMI (kg/m^2)	27.9 ± 5.6	26.6 ± 5.1	28.7 ± 5.8
Smoking (n)	4 (8 %)	2 (11 %)	2 (6 %)
Smoking history (n)	48 (98 %)	18 (100 %)	30 (97 %)
MSQ (0–8, higher is more active)	3.7 ± 2.7	4.1 ± 3.0	3.5 ± 2.6
6MWT			
Distance (meter)	536.2 ± 63.1	556.2 ± 64.3	524.6 ± 60.5
Percent of predicted distance (%)	66.0 ± 20.5	62.1 ± 21.5	68.2 ± 20
Activity tracker			
Number of daily steps	8877.4 ± 5132.2	7937.6 ± 3895.8	9460.7 ± 5756.4
Number of daily active minutes	77.3 ± 51.1	69.8 ± 37.7	82.0 ± 58.0
CCQ (0–6, higher is worse)	2.4 ± 1.2	2.4 ± 1.2	2.5 ± 1.2
mMRC (n)			
mMRC 0 (No impairment)	4 (8 %)	2 (11 %)	2 (6 %)
mMRC 1 (Slight impairment)	11 (22 %)	4 (22 %)	7 (23 %)
mMRC 2 (Moderate impairment)	20 (41 %)	9 (50 %)	11 (35 %)
mMRC 3 (Severe impairment)	10 (20 %)	3 (17 %)	7 (23 %)
mMRC 4 (Very server impairment)	4 (8 %)	0 (0 %)	4 (13 %)
Exacerbations (n)			
Exacerbation 1 (Lung attacks)	21 (43 %)	9 (50 %)	12 (39 %)
Exacerbation 2 (Antibiotic treatment)	20 (41 %)	9 (50 %)	11 (35 %)
Exacerbation 3 (Hospital admissions)	5 (10 %)	3 (17 %)	2 (6 %)
SARC-F (0–10, higher is worse)	3.6 ± 2.8	2.7 ± 1.9	4.1 ± 3.1
MUST (n)			
MUST 0 (No risk for malnutrition)	43 (88 %)	16 (89 %)	27 (87 %)
MUST 1 (Medium risk for malnutrition)	3 (6 %)	0 (0.0 %)	3 (10 %)
MUST 2 (High risk for malnutrition)	3 (6 %)	2 (11 %)	1 (3 %)
GOLD-Stages (n)			
GOLD – 1	0 (0 %)	0 (0 %)	0 (0 %)
GOLD – 2	17 (35 %)	6 (33 %)	11 (35 %)
GOLD – 3	22 (45 %)	7 (39 %)	15 (48 %)
GOLD – 4	8 (16 %)	4 (22 %)	4 (13 %)
KNGF-Profiles (n)			
KNGF – 1	5 (10 %)	2 (11 %)	3 (10 %)
KNGF – 2	18 (37 %)	8 (44 %)	10 (32 %)
KNGF – 3	0 (0 %)	0 (0 %)	0 (0 %)
KNGF – 4	18 (37 %)	4 (22 %)	14 (45 %)
KNGF – 5	7 (14 %)	4 (22 %)	3 (10 %)
SYS-phenotypes (n)			
SYS – 1	19 (38 %)	9 (50 %)	10 (32 %)
SYS – 2	13 (27 %)	2 (11 %)	11 (36 %)
SYS – 3	17 (35 %)	7 (39 %)	10 (32 %)

Data are presented as mean ± SD or n (%).

3.2. GOLD stage is associated with oxylipins, lysophospholipids and bile acids

For GOLD stages, ordinal regression model results reveal that 3 LPSs, 3 oxylipins and 1 sphingolipid are predictors for GOLD stage with positive relationships (all $p < 0.05$; Fig. 2A). Moreover, different metabolites and metabolite ratios are found to vary significantly among the GOLD stages (Fig. 2B) based on the ANCOVA results. It was observed that LPE (18:1) ($p = 0.026$) and DCA ($p = 0.037$) were increased in GOLD stage 4. In the meantime, the 2 oxylipins ratios 16-HDoHE/DHA ($p = 0.046$) and HDoHEs/DHA ($p = 0.042$) display increased abundance with higher GOLD stage, in which the variation of HDoHEs/DHA shares the same trend with ordinal regression results. For these 4 metabolites (LPE (18:1), DCA, 16HDoHE/DHA and HDoHEs/DHA), the plasma of patients from stage 4 exhibits obviously higher concentration compared with other stages. On the contrary, 4 bile acids ratios (GCA/CA, $p = 0.04$; TCA/CA, $p = 0.026$; TDCA/DCA, $p = 0.042$; TCDCA/CDCA, $p = 0.032$) and sphinganine-1-phosphate (18:0) ($p = 0.032$) are more enriched in stage 3 while stage 2 and stage 4 do not show a significant difference.

3.3. KNGF profile is linked to oxylipins and lysophospholipids

The ordinal regression models indicate that increases in LPA (14:0), 8,12-isoiPF2α-VI, GUDCA, and TUDCA were related with higher KNGF profile (all $p < 0.05$; Fig. 3A). As for the ANCOVA results, the targets classified within the same lipid subclass exhibit similar variation patterns among different KNGF profiles (Fig. 3B). For instance, the trend of increase followed by a decrease is found in LPE (16:0) ($p = 0.002$), LPE (18:0) ($p = 0.004$) and LPE (22:4) ($p = 0.043$). Regarding LPA (14:0) ($p = 0.029$) and LPA (16:0) ($p = 0.029$), the abundance of targets is higher in KNGF profile 4 patients' plasma compared to other profiles. In addition, the relative concentrations of LPS (18:0) ($p = 0.002$) and LPS (20:4) ($p = 0.012$) increase in KNGF profile 2 but subsequently decrease in KNGF profile 4 and profile 5, for which trend is also found in sphingosine (18:1) ($p = 0.041$). For the oxylipins, ratios of HEPEs/EPA ($p = 0.025$) and 14-HDoHE/DHA ($p = 0.004$), and PGK2 ($p = 0.041$) exhibit tilde-shape shifting trends from lower to higher profiles except for 8,12-iso-iPF2α-VI ($p = 0.038$), which is constantly increased.

3.4. SYS phenotype is related to specific oxylipin targets and lysophospholipids

Fig. 4A shows that LPS (18:0) and 4 oxylipins (12-HETE, 14-HDoHE, 14HDoHE and 12-HEPE) are positive predictors for higher SYS phenotype (all $p < 0.05$). Meanwhile, ANCOVA analysis displays the variation of signaling lipids in different SYS phenotypes (Fig. 4B). For 13-HOTE ($p = 0.045$), PGF3α ($p = 0.037$), Sphingosine-1-phosphate (16:1) ($p = 0.046$) and thromboxane-B2 ($p = 0.009$), their relative concentrations in phenotype 2 are higher than phenotype 1 and phenotype 3 while LPS (18:0) ($p = 0.027$) shows high abundance in both phenotype 2 and phenotype 3. Moreover, 12-HETE ($p = 0.018$) and 14-HDoHE ($p = 0.038$) exhibit elevated relative concentrations during the transition from phenotype 1 to phenotype 3.

3.5. Correlations between metabolites and classification systems to clinical variables

After exploring the association between signaling lipids and different COPD classifications, the clinical relevance of these lipid targets and COPD was inspected by Spearman correlation analysis (Fig. 5). Regarding physical activity, a negative correlation with 1-OG & 2-OG is observed. KNGF profiles demonstrate a moderate negative correlation with average steps, active minutes, and 6MWT. In addition, LPE (16:0), LPE (18:0), and lipoxin A5 exhibit a positive correlation with physical activity.

Furthermore, the main COPD prognostic factors including sarcopenia, exacerbation, malnutrition and dyspnea were also examined. For sarcopenia, there is a positive correlation to cLPA (20:4), UDCA, KNGF profiles and SYS phenotypes. Regarding exacerbation, a cluster of oxylipins is positively associated with lung attacks and antibiotic treatment, specifically HETEs derived from AA, HEPEs derived from EPA, and HDoHEs derived from DHA. Several lysophospholipids and bile acids show positive correlations with exacerbations, whereas, the remaining bile acids and bile acid ratios negatively correlate with dyspnea. The last section of clinical indicators is Clinical COPD Questionnaires (CCQ), which a self-administered, health-related quality of life questionnaire. LPI (18:2) and 12-HEPE/EPA were found to be weakly associated with the score of emotion. Nevertheless, the KNGF and SYS phenotypes also demonstrate a close correlation with CCQ.

Sample	GOLD	KNGF	SYS
2	2	1	2
7	2	2	1
17	2	2	1
6	2	2	2
10	2	2	2
15	2	2	3
35	2	2	3
8	2	4	1
37	2	4	1
1	2	4	2
29	2	4	2
48	2	4	2
11	2	4	3
32	2	4	3
46	2	4	3
33	2	5	1
9	2	5	3
38	3	1	1
41	3	1	1
45	3	1	1
4	3	2	1
27	3	2	1
49	3	2	1
19	3	2	2
30	3	2	2

Sample	GOLD	KNGF	SYS
14	3	2	3
21	3	2	3
31	3	2	3
39	3	2	3
24	3	4	1
23	3	4	2
44	3	4	2
12	3	4	3
40	3	4	3
42	3	4	3
3	3	5	1
18	3	5	2
36	3	5	3
20	3	NA	3
25	4	2	2
13	4	2	3
26	4	4	1
28	4	4	1
22	4	4	2
43	4	4	3
5	4	5	1
47	4	5	1
34	NA	1	1
16	NA	2	1

Fig. 1. Participants categories in GOLD, KNGF and SYS.
Each participant has been categorized into different groups under GOLD stages, KNGF profiles and SYS phenotypes. The data was sorted in ascending order, first by GOLD stages, then by KNGF profiles. NA represents missing values.

4. Discussion

This study applied a signaling lipid assay to unveil the signaling pathways and mechanisms behind COPD phenotypes. Our results revealed that lysophospholipids, sphingolipids, oxylipins and bile acids are related to different COPD classifications, as summarized in Fig. 6.

Lysophospholipids could be classified as lysoglycerophospholipids and lysosphingolipids based on their chemical backbones. In addition, the lysophospholipids could be further characterized according to their polar head group: lysophosphatidylethanolamine (LPE), lysophosphatidylserine (LPS), lysophosphatidylinositol (LPI), and lysophosphatidic acid (LPA), etc. [49,50]. For LPEs, previous research has shown that LPE (16:0), LPE (18:0) and LPE (18:1) could activate MAPK-ERK1/2 pathway [51,52] which would contribute to mucus overproduction, cytokine expression, apoptosis and fibrosis [53]. Furthermore, LPE (16:0) was reported to be positively related to smoking [54], and LPE (18:0) exhibits a positive correlation with chronic stress and cardiovascular disease risk [55]. Our data showed that LPE (18:1) exhibited an obvious increase in GOLD stage 4 suggesting possible activation of MAPK-ERK1/2 pathway, which is upstream of diverse inflammatory cytokines expressions. Furthermore, LPE (16:0) and LPE (18:0) are increased in KNGF profiles 2 and 4, which are also positively correlated with the percentage of predicted distance and active minutes. Therefore, greater physical activity may lead to an increased production of these two LPEs. Regarding SYS phenotype, there is no evidence to show its association with LPEs.

Apart from LPEs, it has been discovered that LPS and LPA mediated receptors, which are mainly G-protein coupled receptors (GPRs), also play essential roles in inflammatory diseases [56]. Importantly, the LPS receptor is found almost exclusively in immune tissue and immune cells. The activation of LPS receptors would trigger multiple immune processes, such as activation of RhoA-ROCK signaling [57], production of pro-inflammatory cytokines [58], enhancement of mast cell degranulation [59], etc. In our ordinal regression model LPS (18:0), LPS (20:4) and LPS (22:6) were found to be predictors for higher GOLD stages, suggesting they could serve as potential markers for immune system activation. The varied levels of LPS (18:0) and LPS (20:4) were also observed in different KNGF profiles. LPS (18:0) and LPS (20:4) initially increased in profile 2, then decreased in profiles 4 and 5 which may imply that mild impairment triggers immune response whereas severe impairment could contribute to the adjustable adaptation. Similarly, the rise and subsequent decline of LPS (18:0) observed in SYS phenotypes support the same hypothesis proposed for KNGF profiles. Moreover, LPA receptors, LPA_1 and LPA_2 have been reported to participate in the respiratory system and inflammatory response [60]. Elevated LPA levels in the bronchial lavage fluid were observed in an acute lung injury (ALI) model [61], whereas knockdown of LPA_1 exerted significant protection effects against inflammation [62]. Only the KNGF profile exhibited the association with the LPA cluster (LPA (14:0), LPA (16:0)). However, further exploration is required due to the complex trend from profile 1 to profile 5.

Oxylipins are oxidation products of polyunsaturated fatty acids (PUFAs), such as linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). A number of

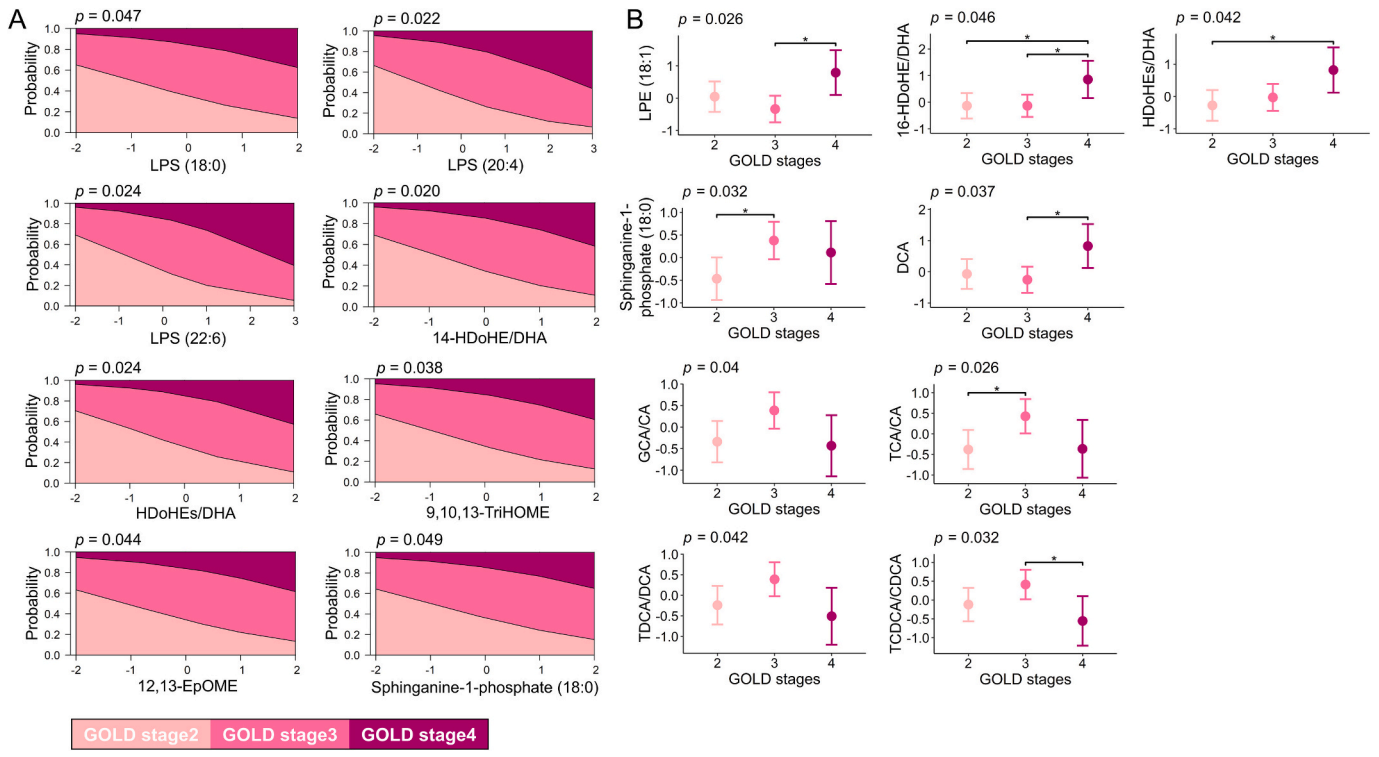

Fig. 2. Signaling lipids associated with COPD GOLD stages.
The results of the Ordinal Regression Model revealed the contribution of signaling lipids to the GOLD stage classification. (B) The results of the Analysis of Covariance indicated the variation of signaling lipids among the GOLD stages. All the targets have been examined in Ordinal Regression Model and Analysis of Covariance, but only targets with p value <0.05 were displayed. Ordinal Regression Model is presented by the probability distribution of different GOLD stages, indicating the likelihood of each patient being in different GOLD stages based on metabolite changes. Analysis of Covariance is presented by estimated marginal mean with the high and low end of the confidence interval. *$p < 0.05$, **$p < 0.01$.

oxylipins have been reported to play a role in COPD and other respiratory diseases. Wang and coworkers reported increased 12-HETE in serum of COPD patients compared to non-COPD patients [63], moreover, 12-HETE and 12-HEPE were increased in serum after exposure to ambient air particulate matter [64]. In addition, many studies verified the importance of 12-LOX, a catalytic enzyme involved in the production of 12-HETE, 12-HEPE and 14-HDoHE, in pulmonary disorders. 12/15-LOX-deficient mice displayed augmented IL-33-induced lung inflammation while the application of 14-HDoHE suppressed airway inflammation in which 14-HDoHE serves as a precursor of an anti-inflammatory regulator, maresin-1 [65]. It is shown that 14-HDoHE/DHA is associated for both GOLD stage and SYS phenotype with a positive relationship, showing an increase in higher KNGF profiles. The other two 12-LOX products, 12-HETE and 12-HEPE were found to increase in higher SYS phenotype. Additionally, these 12-LOX products, along with the total levels of HETEs, HEPEs and HDoHEs exhibited a clear correlation with lung attack and antibiotic treatment. HETEs are considered as indicators of systemic inflammation [66] while HEPEs and HDoHEs are less investigated. The worsening of airway inflammation is the primary evoking event for COPD exacerbation [67]. Additionally, the accuracy of discriminating COPD patients with exacerbations using these targets was also assessed, with 14-HDoHE/DHA demonstrating the best performance (AUC = 0.843), shown in Fig. S2. Thus, 14-HDoHE/DHA may serve as a marker of airway inflammation during exacerbations, potentially assisting in the assessment, monitoring, and subsequent treatment adjustments for COPD patients.

Apart from the oxidation products of 12-LOX, metabolites from cyclooxygenase (COX) and cytochrome P450 (CYPs) pathways also exert functions in pulmonary dysfunction. 12,13-EpOME is produced by inflammatory leukocytes. High levels of EpOMEs were observed in acute respiratory distress syndrome (ARDS) patients [68] and in a female-dominated phenotype of COPD. Upregulated 9,10,13-TriHOME was found in asthmatics following provocation compared with healthy controls [69]. 12,13-EpOME and 9,10,13-TriHOME are derived from LA and discovered to be positive predictors for a higher GOLD stage in this study. However, their roles in COPD remain unclear. Another special oxylipin, TXB2, is involved in platelet aggregation and vasoconstriction. TXB2 is the inactive form of TXA2, yet it could be regarded as an indicator of TXA2 generation [70]. TXA2 was found to increase in allergic asthma subjects' urine while the therapeutic effects were also confirmed with inhibition of TXA2 receptor [71]. Our study found that TXB2 increases in SYS phenotype 2 and 3, which may indicate the dysregulation of pulmonary vessels and airway smooth muscles.

The last cluster of lipids discovered from analyses is bile acids. Bile acids are derived from 7/27-hydroxycholesterol, the primary bile acids are formed in liver including CA and CDCA, then dehydroxylated into secondary bile acids DCA and UDCA/LCA in the intestine by microbiome [72]. To increase the solubility, these bile acids are conjugated with either glycine (G) or taurine (T). Therefore, both ratios of secondary bile acids and the ratios of bile acid conjugates to their corresponding prototypes are investigated. Among these bile acids, CA, CDCA and DCA are activators for Farnesoid X receptor (FXR) while UDCA is an antagonist [73]. The overexpression of FXR leads to airway remodeling and inflammation in COPD via epithelial-mesenchymal transition [74]. Contradictorily, anti-inflammation effects of FXR activation were observed in ALI/ARDS. The administration of an FXR agonist inhibits the release of proinflammatory cytokines and NF-κB pathway [75]. In this study, the bile acids related with GOLD stages are all FXR agonists (conjugates of CA, DCA and CDCA), while the bile acids positively linked to KNGF profiles are all FXR antagonists (conjugates of UDCA). Hence, GOLD stage and KNGF profile may reflect the opposing effects in FXR.

Of all the detected lipids, certain targets showed increased levels in

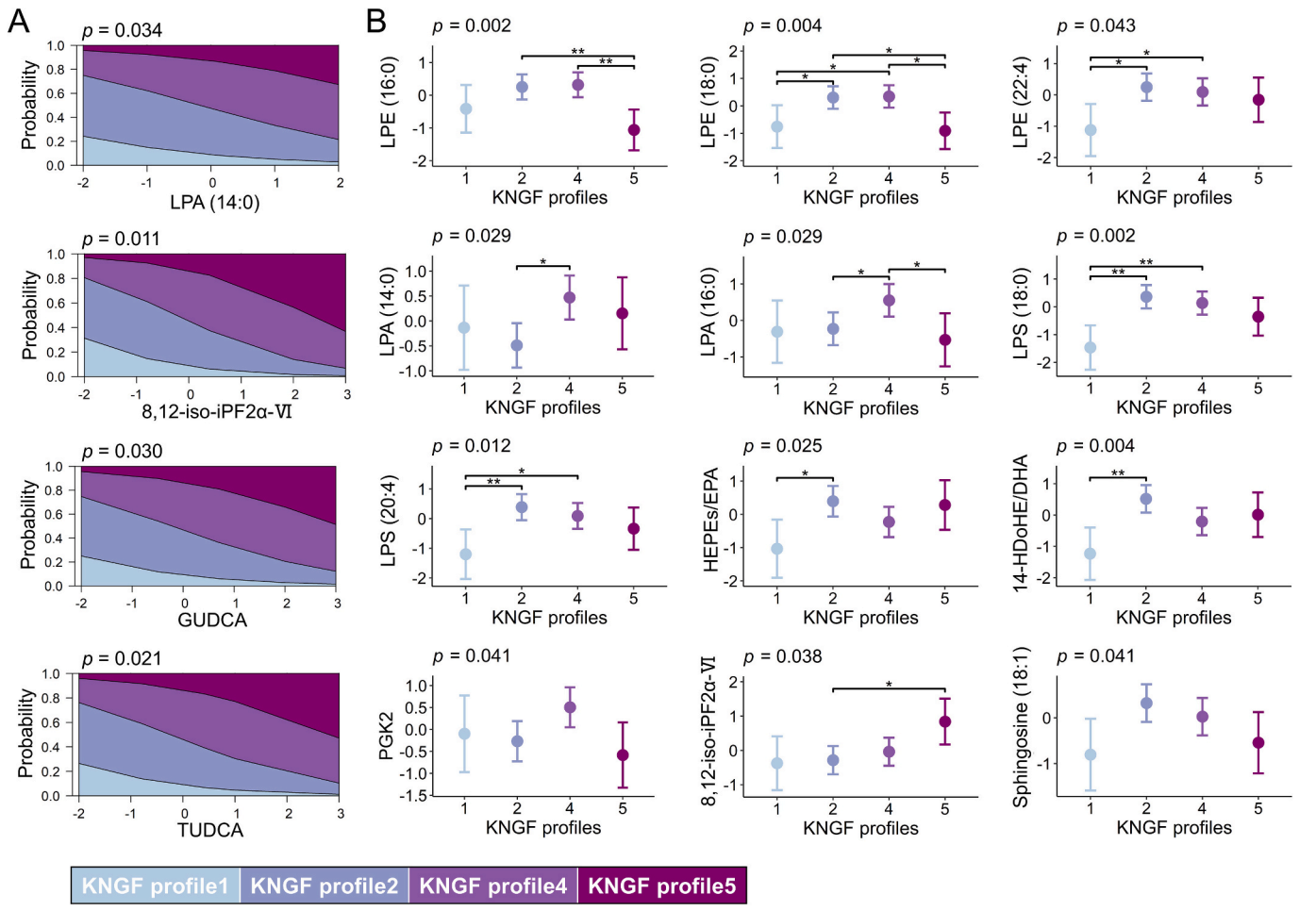

Fig. 3. Signaling lipids associated with COPD KNGF profiles.
The results of the Ordinal Regression Model revealed signaling lipids contribution to the KNGF profile classification. (B) The results of the Analysis of Covariance indicated the variation of signaling lipids among the KNGF profiles. All the targets have been examined in Ordinal Regression Model and Analysis of Covariance, but only targets with p value <0.05 were displayed. Ordinal Regression Model is presented by the probability distribution of different KNGF profiles, indicating the likelihood of each patient being in different KNGF profiles based on metabolite changes. Analysis of Covariance is presented by estimated marginal mean with the high and low end of the confidence interval. *$p < 0.05$, **$p < 0.01$.

the middle classifications compared to lower or higher classifications in GOLD, KNGF and SYS. Four bile acids ratios GCA/CA, TCA/CA, TDCA/DCA and TCDCA/DCA were higher in GOLD stage 3 than in stages 2 and 4. KNGF profiles 2 and 4 showed elevated levels of LPE (16:0), LPE (18:0), LPS (18:0) and LPS (20:4) compared to other profiles. For SYS phenotypes, relative concentrations of 13-HOTE, PGF3α and Sphingosine-1-phosphate (16:1) increased in SYS phenotype 2. KNGF profiles were closely associated with lysophospholipids, while SYS phenotypes showed more variance in oxylipins. Nevertheless, GOLD stages showed connections with all 3 lipids clusters (lysophospholipids, oxylipins and bile acids). In KNGF profile, physical capacity and activity are the main criteria to distinguish profiles 2, 3, 4, and 5. A recent study showed that exercise altered lipid metabolism including phospholipids and lysophospholipids [76], with intensified running leading to a significant decrease in LPE [77], suggesting that KNGF profiles may reflect energy metabolism. SYS phenotypes emphasize systemic symptoms beyond the pulmonary system, with oxylipins playing essential roles in multiple diseases such as cardiovascular diseases, oncological diseases, diabetes, obesity, liver disease, neurological disorders, kidney diseases, and so on [78]. Consequently, SYS phenotypes may be related to systemic conditions. As for GOLD stage, it is determined by lung capacity characterized by FEV1/FVC. The lung capacity can be influenced by physical activity [79], which may account for the association with lysophospholipids [76]. Moreover, HDoHEs and FXR (target of bile acids) exert essential roles in airway inflammation regulation, a key factor affecting lung function. Consequently, the variety of lung function factors leads to the complexity of GOLD stage.

Distinct pathways associated with identified lipid clusters suggest potential connections to inflammation regulation. GPRs, which are receptors for lysophospholipids, represent the largest family of membrane receptors and regulate various signaling pathways, including ERK1/2, p38, MAPK, COX-1/2, and LOX [80]. Notably, oxylipins, as products of LOX and COX, may serve as markers for GPR activation and related inflammatory signaling cascades. In contrast, NF-κB pathway is regulated by inflammasome receptors [81] rather than GPRs. And bile acids can influence the NF-κB pathway and downstream NLRP3 inflammasome [82]. This distinct mechanism may explain why negative correlations with dyspnea were observed only for certain bile acids, indicating that bile acids may contribute to COPD in a different manner.

This study has several limitations. Participant recruitment was more difficult than expected mainly due to the COVID-19 measures that were taken in the Netherlands. Therefore, fewer participants were recruited than anticipated, which may lead to a small sample size and consequently low statistical power, diminishing the significance of the results. Another potential limitation is the lack of data on participants' comorbidities, which could influence metabolism and potentially act as covariates. Furthermore, the male-to-female sex ratio is 58 males to 100 females in this study while the global prevalence of COPD is significantly

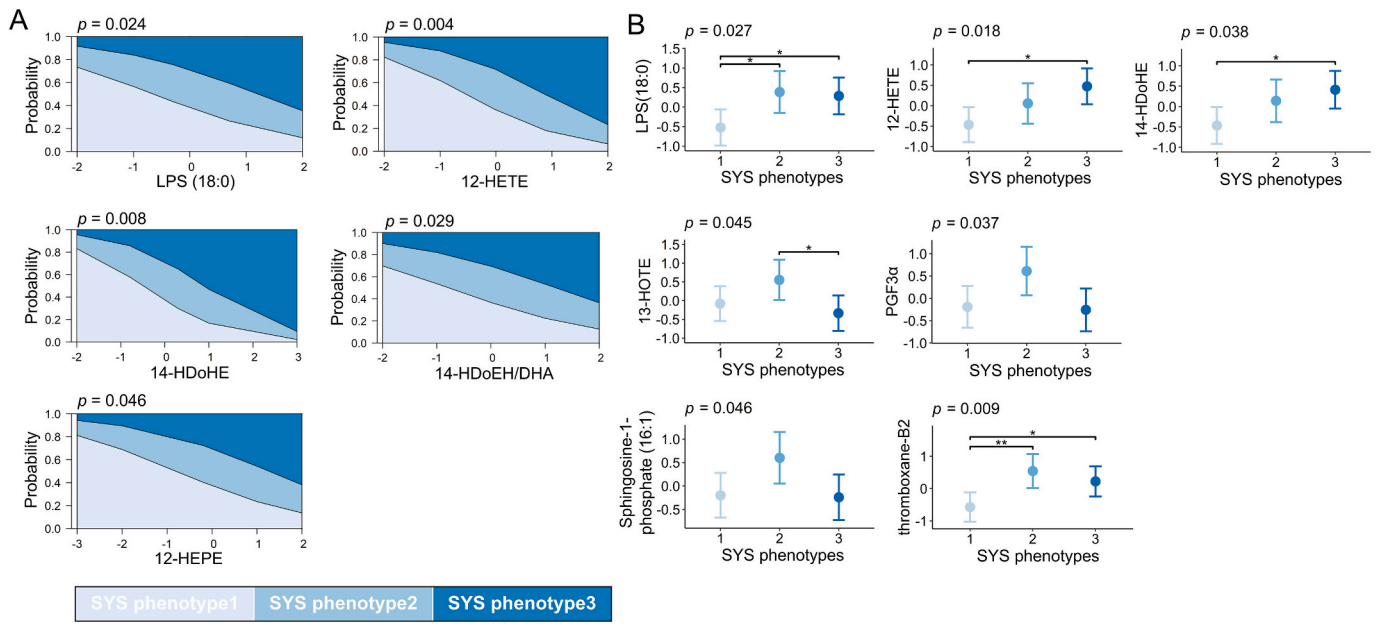

Fig. 4. Signaling lipids associated with SYS phenotypes.
The results of Ordinal Regression Model revealed signaling lipids contribution to the SYS phenotypes. (B) The results of Analysis of Covariance exhibited the variation of signaling lipids among the SYS phenotypes. All the targets have been examined in Ordinal Regression Model and Analysis of Covariance, but only targets with p value <0.05 were displayed. Ordinal Regression Model is presented by the probability distribution of different SYS phenotypes, indicating the likelihood of each patient being in different SYS phenotypes based on metabolite changes. Analysis of Covariance is presented by estimated marginal mean with the high and low end of the confidence interval. $^{*}p < 0.05$, $^{**}p < 0.01$.

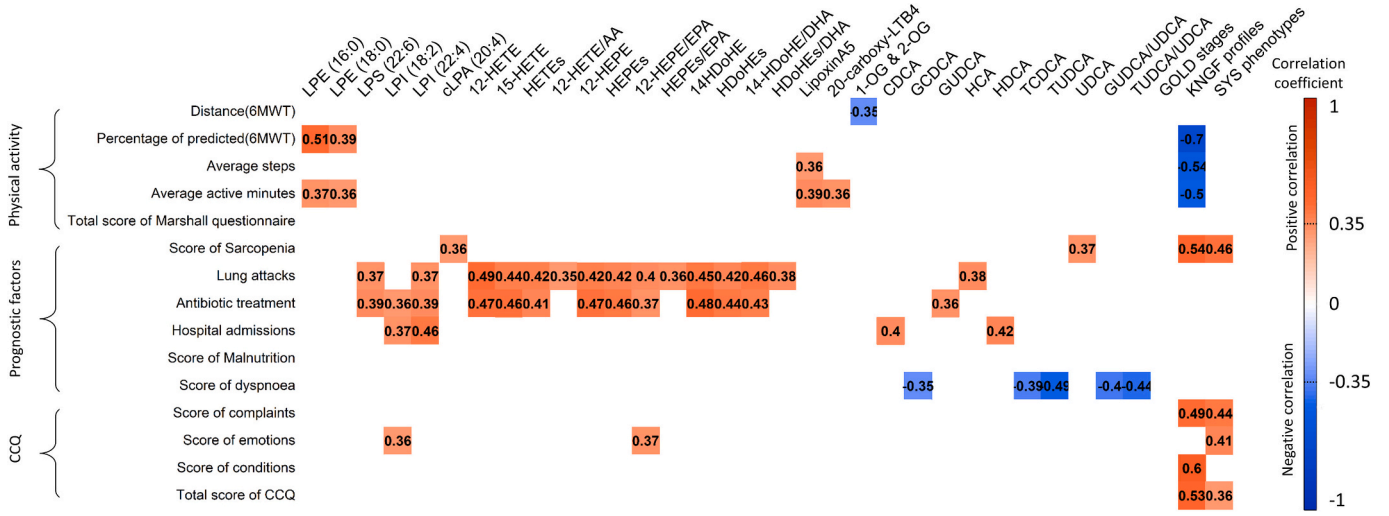

Fig. 5. Spearman correlation analysis between COPD-related clinical indicators with signaling lipids and COPD classifications.
All the targets have been examined but only targets with p value <0.05 were displayed. The digit in the box indicates the correlation coefficients while the blank represents no significant correlation. Blue and red indicate positive and negative correlations respectively.

higher among men (267.4 million) compared to women (124.6 million). That might reduce the applicability of these findings to the real population, particularly among male COPD patients. In addition, the algorithm to calculate the SYS phenotypes was designed in close discussion with a TCM expert, but the resulting algorithm was not validated by additional TCM experts. Therefore, further validation by external TCM experts and additional datasets is needed for improving the reliability of algorithm and findings. Furthermore, while the specific metabolites and related pathways were identified, the causal relationships and how metabolites exactly act in different COPD categories remain unclear in this study.

5. Conclusion

In conclusion, this study applied a metabolomics approach to investigate key metabolites and potential signaling pathways associated with COPD classifications and their possible clinical relevance. The results suggested that specific lipid clusters, i.e. lysophospholipids, oxylipins and bile acids are related to GOLD stages, KNGF profiles, and SYS phenotypes in different ways. These results indicate that the three classification systems are at least partly related to different metabolic mechanisms and therefore indicate different aspects of COPD which might contribute to the development of a more personalized treatment of patients with COPD.

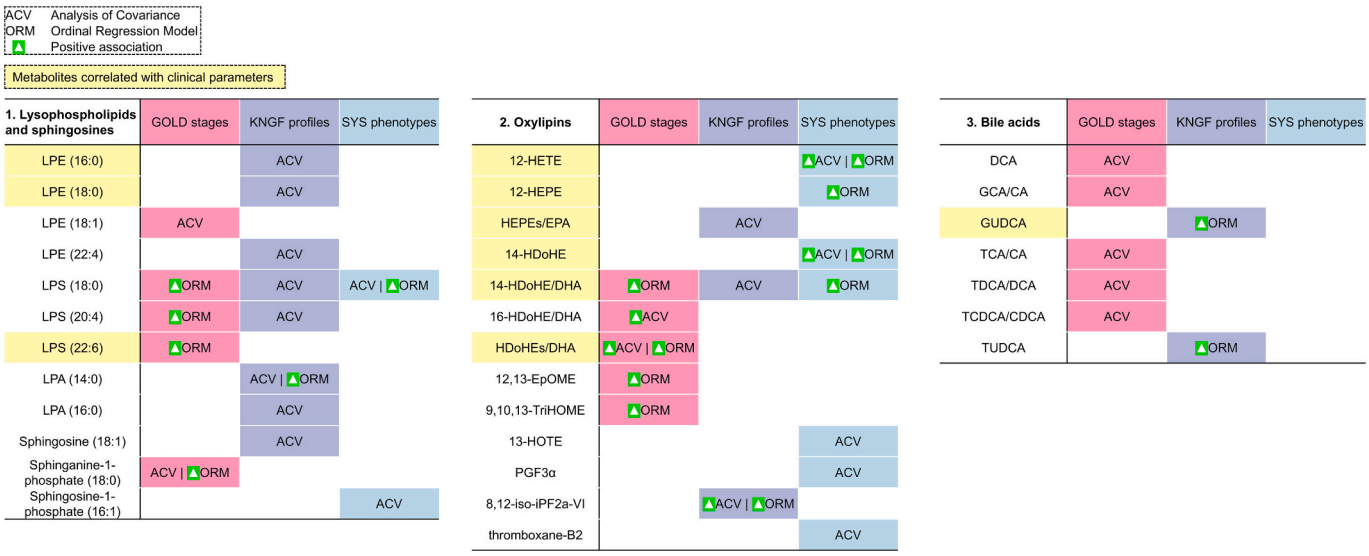

Fig. 6. Summary of COPD classifications related to signaling lipids.
The targets identified by statistical analyses are listed per lipid category. The abbreviation indicates in which statistical method this target exhibited statistical significance. ACV for Analysis of Covariance, and ORM for Ordinal Regression Model. The highlighting of targets presents correlation with clinical indicators.

CRediT authorship contribution statement

Lu Zhang: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Jean Marie Wernet:** Visualization, Investigation, Formal analysis. **Andreas Rothgangel:** Writing – review & editing, Resources, Project administration, Investigation, Conceptualization. **Susy Braun:** Writing – review & editing, Investigation. **Darcy Ummels:** Writing – review & editing, Investigation. **Emmylou Beekman:** Writing – review & editing, Investigation. **Tanja de Jong-van Luxzenburg:** Writing – review & editing, Investigation. **Martijn D. de Kruif:** Writing – review & editing, Resources. **Wei Yang:** Investigation. **Lieke Lamont:** Writing – review & editing, Supervision. **Alida Kindt:** Writing – review & editing, Supervision. **Thomas Hankemeier:** Supervision, Resources. **Amy Harms:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Herman van Wietmarschen:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2025.123438.

Data availability

The data generated and analyzed during the current study are available in the MetaboLights repository under the accession number MTBLS9119.

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