Tryptophan catabolism in acute exacerbations of chronic obstructive pulmonary disease

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Introduction: Exacerbations are a leading cause of morbidity in COPD. The objective of this study was to identify metabolomic biomarkers of acute exacerbations of COPD (AECOPD).

Methods: We measured metabolites via mass spectrometry (MS) in plasma drawn within 24 hours of admission to the hospital for 33 patients with an AECOPD (day 0) and 30 days later and for 65 matched controls. Individual metabolites were measured via selective reaction monitoring with mass spectrometry. We used a mixed-effect model to compare metabolite levels in cases compared to controls and a paired *t*-test to test for differences between days 0 and 30 in the AECOPD group.

Results: We identified 377 analytes at a false discovery rate of 5% that differed between cases (day 0) and controls, and 31 analytes that differed in the AECOPD cases between day 0 and day 30 (false discovery rate: 5%). Tryptophan was decreased at day 0 of AECOPD compared to controls corresponding to an increase in indoleamine 2,3-dioxygenase activity.

Conclusion: Patients with AECOPD have a unique metabolomic signature that includes a decrease in tryptophan levels consistent with an increase in indoleamine 2,3-dioxygenase activity.

Keywords: Chronic Obstructive Pulmonary Disease, metabolomics, tryptophan

Introduction

Chronic obstructive pulmonary disease (COPD) is currently the third leading cause of death in the USA, and worldwide it is one of the most prevalent lung diseases causing significant morbidity and mortality.^{1,2} Some patients are prone to episodes of acute exacerbations of COPD (AECOPD) that are a frequent cause of medical visits and hospitalizations. AECOPD are a leading cause of morbidity and mortality in COPD, and there is evidence that frequent exacerbations might accelerate the natural course of COPD.³⁻⁶ Currently, there is no validated diagnostic test or biomarker to identify patients at risk of or with an exacerbation. Therefore, a diagnosis of an AECOPD is based on clinical symptoms for which there are no single standardized definition.

Recent technological advances in mass spectrometry have led to the emerging field of metabolomics, the study of small molecules. These small molecules consist of metabolic substrates and products, such as lipids, sugars, peptides, and foreign compounds such as drugs and their metabolites. Metabolomic profiling complements genomics and proteomics offering a snapshot into the physiology of human disease. In this respect, metabolomics has the opportunity to give us insight into mechanisms of disease and has the potential to identify biomarkers of disease.

In this study, we profiled the plasma metabolome in participants from the NIH-sponsored COPD Clinical Research Network. The cases were participants hospitalized for an AECOPD who had plasma collected within 24 hours of hospitalization and 30 days into

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recovery. Controls consisted of COPD participants matched for age and lung function in the absence of an AECOPD. We were able to characterize metabolomic profiles that distinguish an AECOPD and the presence of tryptophan catabolism via indoleamine 2,3-dioxygenase (IDO) activation.

Methods

Study population

Subjects (Table 1) were 33 individuals with COPD (defined as having forced expiratory volume in 1 second [FEV₁] <60% predicted, FEV₁ to forced vital capacity ratio <70%, and minimum 10 pack-years of smoking) hospitalized for an AECOPD in the NIH-sponsored LEUKO study.⁷ Plasma was collected within 24 hours of hospitalization (day 0) and then again in 30 days post-AECOPD (day 30). Controls (n=65) consisted of individuals with stable COPD from the NIH-sponsored MACRO study⁸ matched for age, sex, lung function, and pack-years of smoking. Participants in the MACRO study were considered high risk for exacerbation as they were either using continuous oxygen therapy or had received systemic glucocorticoid steroids, had gone to the emergency room or been hospitalized for an AECOPD within the previous year. Samples from controls were obtained at baseline, prior to randomization, and subjects had not been treated for AECOPD for at least 4 weeks at the time of the plasma collection. Two controls were matched to each case for age and lung function (FEV₁) with the exception of one case with only one matched control identified. This study was approved by the University of Minnesota Institutional Review Board and met exempt status for patient consent.

Sample preparation

Both cases and controls had identical protocols for obtaining plasma. These were nonfasting samples drawn at

the time of enrollment into the study. Briefly, blood was drawn into an ethylenediaminetetraacetic acid-containing tube, inverted 8–10 times, and then centrifuged. Following centrifugation, 1.0 mL of plasma was transferred to a microtube (Sarstedt [Nümbrecht, Germany], RNase, and DNase free) and immediately frozen to –70°C. The samples remained at –70°C until use and were not freeze-thawed. Plasma samples were processed using a high-performance liquid chromatography-grade cold methanol (Sigma-Aldrich Co, St Louis, MO, USA) extraction method. The extraction methanol solution was cooled to –80°C. A volume of 400 µL of cold solvent was added to 100 µL of plasma. The mixture was gently shaken for 30 seconds and incubated for 6–8 hours at –20°C, then centrifuged for 15 minutes at 13,000 rpm at 4°C (in a cold room) and the supernatant transferred to a new tube. The pellet was rinsed twice with the cold solvent and the aforementioned procedure was repeated. The resulting supernatants were pooled and dried with a SpeedVac and stored at –80°C until further processing. A volume of the starting ultra-performance liquid chromatography (UPLC) buffer was added to the dried samples after they were acidified with formic acid (5 µL of formic acid [50% v/v]), to which 95 µL of the UPLC starting buffer was added (5% acetonitrile, 94.9% water, and 0.1% formic acid). After the samples were reconstituted, the solutions were centrifuged, to pellet out insoluble material, for 5 minutes at 13,000 rpm (4°C), and the supernatants were transferred to a Waters (Waters, Milford, MA, USA) 300 µL polypropylene plastic vial. For selective reaction monitoring (SRM) analysis, 100 µL of sample was added to 3 µL 100 µm kynurenine D6 and 3 µL 1 mm tryptophan ¹³C11 (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) prior to protein precipitation. Samples were vacuum-dried and diluted to 10^{–3} for tryptophan and 10^{–2} for kynurenine with load buffer.

Table 1 Characteristics of case and control subjects

Characteristics	Cases day 0 (N=33)	Cases day 30 (N=33)	Controls (N=65)	P-value
Age, years	62.03 (51–78)	–	62.57 (50–81)	0.1
Sex: male	18 (57%)	–	38 (58%)	1
FEV ₁ percent predicted	31.18 (14.9–75.2)	–	34.49 (14.3–72.5)	0.07
Pack years	47.92 (11–150)	–	44.90 (14–144)	0.23
Beta agonist	22 (0.67)	23 (0.7)	51 (0.78)	0.42
Methacholine antagonist	15 (0.45)	7 (0.21)	21 (0.32)	0.11
LABA	22 (0.67)	24 (0.73)	48 (0.74)	0.77
LAMA	21 (0.64)	18 (0.55)	46 (0.71)	0.28
ICS	23 (0.7)	24 (0.73)	48 (0.74)	0.94
Steroids	13 (0.39)	3 (0.09)	0 (0)	<0.01
Antibiotic	17 (0.52)	6 (0.18)	0 (0)	<0.01

Notes: For continuous variables, the mean and the range are presented (the latter in parentheses) and for binary variables the count and the percent are provided (the latter in parentheses). The P-value tests the null hypothesis of no difference among the three groups and is obtained from a linear mixed-effects model for continuous variables and from a generalized linear mixed-effects model with logistic link for binary variables.

Abbreviations: LABA, long-acting beta-agonist; LAMA, long-acting methacholine antagonist; ICS, inhaled corticosteroid; FEV₁, forced expiratory volume in 1 second.

UPLC-MS analysis

For UPLC-MS^e analysis, a Waters Acquity UPLC coupled to a Waters Synapt G2 HDMS quadrupole orthogonal acceleration time of flight mass spectrometer was used. A Waters Acquity BEH C18 2.1×100 mm column (1.7 µm diameter particles) at 35°C was used during the following 26 minutes gradient separation with A: Water containing 0.1% formic acid; B: high-performance liquid chromatography grade acetonitrile (Fisher Scientific, Pittsburg, PA, USA) containing 0.1% formic acid, at a flow rate of 0.4 mL/minute: 3% B, 0–3 minutes; 3% B–97% B, 3–18 minutes; 97% B, 18–21 minutes; 97% B–3% B, 21–23 minutes; 3% B 23–26 minutes. Simultaneous low- and high-collision energy (CE) mass spectra were collected in centroid mode over the range mass/charge (*m/z*) 100–1,200 every 0.1 second during the chromatographic separation. MS^e parameters in positive electrospray ionization mode were as follows: capillary, 0.30 kV; sampling cone, 35.0 V; extraction cone, 4.0 V; desolvation gas flow, 800 L/hour; source temperature, 100°C; desolvation temperature, 350°C; cone gas flow, 20 L/hour; trap CE, off (low CE collection), ramp 15–65 V (high CE collection); Lockspray configuration consisted of infusion of a 5 µg/mL solution of leucine-enkephalin (Waters); and acquisition of one mass spectrum (0.2 second scan, *m/z* 100–1,200) every 10 seconds. Three lockspray *m/z* measurements of protonated leucine-enkephalin were averaged and used to apply corrections to measured *m/z* values during the course of the analysis. The R software package RAMClustR was used for analyte alignment and feature detection.⁹

SRM analysis of tryptophan and kynurenine

Samples (10 µL) for SRM analysis were subjected to injection using an Agilent autosampler with an analytical Acquity UPLC BEH C18, 1.7 µm, 2.1×50 mm column fit with an Acquity UPLC BEH shield RP18 precolumn connected to the Applied Biosystem 5500 iontrap fit with a turbo V electrospray source. The samples were subjected to a linear gradient of 2% acetonitrile, 0.1% formic acid to 98% acetonitrile 0.1% formic acid for 10 minutes at a column flow rate of 250 µL/minute. Transitions monitored are listed in Table S1, and these were established using the instrument optimization mode with direct injection of native and heavy tryptophan and kynurenine. The data were analyzed using MultiQuantTM (ABI Sciex, Framingham, MA, USA), which provided the peak area ratio of tryptophan/tryptophan ¹³C11 and kynurenine/kynurenine D6 for the transitions. A standard curve was constructed using concentration ratios of tryptophan/tryptophan

¹³C11 and kynurenine/kynurenine D6 (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) from picomole to nanomole in 10 µL. Samples were run in duplicate and concentrations were determined from the standard curve. The correlation across duplicates for tryptophan was 0.9839 and for kynurenine was 0.9589.

Statistics

The processed data from the MS experiments were transformed by adding 1 to all data points and taking the logarithm as the marginal distributions of the feature data were positively skewed (1 was added as many zeroes were observed in the data). To test for differences between cases and controls, a mixed-effects model was used with random effects for cluster membership (a case plus its two matched controls formed a cluster) and fixed effects for case–control status. The *p*-values from the test of no group effect were then adjusted for multiple comparisons using the method of Storey, and a false discovery rate (FDR) of 0.05 was used to select features for further investigation.¹⁰ To test for differences between day 0 and day 30 among the cases, a paired *t*-test was used and adjustments for multiple hypothesis testing were conducted in the same manner as the test for differences between cases and controls. For the analysis of the data arising from the SRM experiments, a single mixed-effects model was fit that allowed testing for differences between cases and controls and for changes from day 0 to day 30 for tryptophan and kynurenine and their ratio. No adjustment was made for multiple hypotheses testing after fitting these models. These models also included the effects of sex, age, pack-years of smoking, lung function, and medications (steroids and antibiotics) as fixed effects and case–control group and subject as random effects (with subject effects nested within the case–control group effects) for the SRM experiments.

Results

Characteristics of study participants

We analyzed two longitudinal plasma samples from 33 individuals with a COPD exacerbation who were recruited as part of the LEUKO study. Each subject had a plasma sample obtained within the first 24 hours of being hospitalized for a COPD exacerbation and a follow-up plasma sample obtained 30 days later. Controls consisted of individuals at high risk of developing AECOPD, but were currently free from an exacerbation (Table 1). All subjects had at least a ten pack-year history of smoking, with 27% of cases and 24% of controls reporting active smoking at the time of enrollment. The FEV₁ ranged in severity from moderate to very severe according

to the GOLD classification (GOLD II–IV), with the average FEV₁ in the GOLD class III. The majority of subjects were on long- and short-acting β -agonists. The main difference in medications was more steroid and antibiotic use in the day 0 group compared to both day 30 and controls.

Analyte profiles

We detected over 3,000 analyte signals in the plasma. An analyte refers to a discrete m/z and retention time on the mass spectrometer that correlates with a yet unknown metabolite. Currently, there is no accepted methodology to quantify analytes detected by mass spectrometry. For our study, relative abundance was measured as the sum of all peak intensities detected by the mass spectrometer that associated with the given analyte. Using a mixed-effect model to account for the pairing of multiple controls to cases, we identified 583 analytes at 10% FDR and 386 analytes at a 5% FDR that were significantly different between samples at day 0 (cases) and controls. Using a paired t -test, we detected 54 analytes at 10% FDR and 34 analytes at a 5% FDR that were significantly different between samples at day 0 and day 30. A search within the Metlin library identified that several of the analytes found were consistent with the medications zileuton and prednisolone. These medications were anticipated since zileuton was the interventional drug administered in the LEUKO trial and since treatment with steroids is a common practice in an AECOPD. No other medications were identified. These analytes were eliminated, leaving 31 and 379 analytes at 5% FDR in the two groups (day 0 vs day 30 and controls vs day 0), respectively (Tables S2 and S3). We found considerable overlap in the analytes between the two groups as depicted in the Venn diagram (Figure 1). Of the 23 analytes

in common between the two groups, nine are consistent with small peptides consisting of 3–4 amino acids and three are consistent with lipids (Table S2). Figure 2 demonstrates 25 representative analytes that are differentially expressed comparing day 0 to day 30, plus values for their respective controls. This figure demonstrates that the pattern of analytes show a similar value comparing day 0 and controls.

Tryptophan catabolism

One of the analytes differentially expressed in day 0 subjects compared to controls was consistent with the essential amino acid tryptophan (Trp, m/z 204.23 and 257.09 methoxytryptophan). Since tryptophan catabolism has been associated with both immune modulation and infection, we sought to quantify tryptophan and its major metabolite, kynurenine. To identify tryptophan and measure its concentration, we performed SRM. IDO is the main inducible and rate-limiting enzyme involved in tryptophan catabolism, with kynurenine as the main metabolite of the IDO pathway. IDO activity is expressed as a ratio of kynurenine to tryptophan (Kyn/Trp). Statistical models included controlling for the effects of sex, age, pack years, lung function, and medications (steroids and antibiotics). We found that tryptophan was lower at day 30 compared to day 0 and higher in controls than day 0, but this was not statistically significant after controlling for the potential confounders (Figure 3). We did find that kynurenine levels were significantly lower at day 30 compared to day 0 ($P=0.00292$, Figure 3). With respect to IDO activity as measured by the Kyn/Trp ratio, Kyn/Trp was higher at day 0 compared to day 30 ($P=0.0352$) and higher at day 0 than in controls ($P=0.0338$, Figure 4).

Discussion

Patients with COPD often experience exacerbations, and, currently, there is no biomarker that either predicts or identifies those with an exacerbation. In this study, we identified a plasma metabolomic biosignature in COPD patients with an acute exacerbation. The largest profile was seen in COPD patients with an AECOPD (day 0) compared to matched controls. A smaller biosignature was identified in day 0 compared to day 30, and many of these analytes overlapped with the larger profile. This smaller biosignature suggests that full recovery from the exacerbation may not yet exist by day 30. This is not a surprise since one in eleven COPD patients are readmitted within 30 days following hospitalization.¹¹ Therefore, full recovery following a severe exacerbation may take longer than 30 days. As expected among these analytes, zileuton and prednisolone were identified. Zileuton was the

Figure 1 Venn diagram that depicts the number of analytes overlapping between the two comparison groups.

Figure 2 Analyte expression.

Notes: 25/34 of the analytes identified at 5% FDR that were differentially expressed comparing day 0 and day 30. Top number is retention time and bottom number is *m/z*. X-axis: C = control, d0 = day 0, d30 = day 30. Y-axis: peak intensity.

Abbreviations: FDR, false discovery rate; *m/z*, mass/charge.

parent trial study drug, and patients are often immediately placed on prednisone upon admission for an AECOPD.

One of the analytes in the profile comparing day 0 to controls was consistent with tryptophan. We used SRM to accurately measure tryptophan and its main metabolite kynurenine. We found that tryptophan levels are reduced early in the course of an AECOPD (day 0) compared to “healthy” COPD patients. This decrease in tryptophan is consistent with an increased catabolism through the IDO pathway as demonstrated by an increase in Kyn/Trp. After

30 days of recovery from an AECOPD, tryptophan levels remained significantly lower compared to control subjects; however, IDO activity was no longer increased at that time. This suggests that tryptophan catabolism was decreasing by day 30, but was incomplete. In this study, longitudinal samples were limited to 30 days; therefore, we do not know whether tryptophan levels eventually normalized, similar to controls.

Tryptophan is an essential amino acid and its deficiency limits protein synthesis, resulting in cellular dysfunction

Figure 3 Tryptophan and kynurenine expression.

Notes: No statistically significant differences in tryptophan levels. Kynurenine levels were significantly lower at day 0 compared to day 30 ($P=0.00292$).

and decreased proliferation. Teleologically, it is felt that tryptophan catabolism is beneficial during infection, where a decline in tryptophan levels inhibits bacterial proliferation. Recent studies have also implicated tryptophan catabolism through the IDO pathway as having antimicrobial effects. The list of pathogens sensitive to tryptophan catabolism via IDO includes respiratory pathogens common in AECOPD such as *Streptococci*.¹² A decrease in serum tryptophan levels has been reported in pulmonary infections and predicts prognosis

in both tuberculosis and community-acquired pneumonia.^{13,14} Since AECOPD is often due to respiratory tract infections, it is a possibility that tryptophan catabolism in that setting is actually a biomarker for infection.

Tryptophan catabolism is also an important factor in the lung microenvironment that influences immune responses. Tryptophan catabolism occurs predominantly through the activation of the enzyme IDO,^{15,16} producing metabolites of the kynurenine pathway. Most of the effects of tryptophan catabolism come from accumulation of its active metabolites, such as kynurenine, rather than tryptophan depletion.^{15,16} The generation of kynurenine through IDO activation leads to immune tolerance and an anti-inflammatory effect through the proliferation of T_{reg} FoxP3 cells and suppression of Th17 cells.^{16,17} The immune tolerance effect of IDO activation has been implicated in lung cancer and HIV infection.^{18–22} Thus, tryptophan depletion and IDO activation have both antimicrobial and anti-inflammatory effects.¹²

Although decreases in tryptophan and IDO activation have been reported in lung cancer and certain lung infections, little is known of its role in COPD. Perturbations in amino acids in both serum and exhaled breath have been described in COPD using mass spectrometry and NMR.^{23,24} Ubhi et al²⁴ measured amino acid metabolism using mass spectrometry in COPD patients from the ECLIPSE cohort and found tryptophan levels were decreased in the serum of COPD patients with emphysema.²⁴ However, they did not assess IDO activity. However, Maneechotesuwan et al²⁵ found IDO activity decreased in the sputum of COPD patients that correlated with severity of disease and a reversal between the IL-10 and IL-17A balance. This suggests that a decrease in IDO activity within sputum creates an environment supporting neutrophilic inflammation.²⁵ In our study, we found tryptophan levels to be decreased in the plasma of patients with an AECOPD consistent with an activation of IDO, as measured by kynurenine and tryptophan ratios. This increase in IDO activity was still present at day 30, but to a lesser extent. A decrease in tryptophan would have an antimicrobial effect that would be beneficial in AECOPD, along with an anti-inflammatory effect to mitigate airway injury. The role of tryptophan catabolism in COPD and possible link to lung cancer remains unknown.

Many of the analytes that were common between the two biosignatures were multiply charged and had a retention time consistent with peptides consisting of 2–4 amino acids. Peptides as biomarkers for lung disease is not a new concept – over 30 years ago, Kucich et al²⁶ detected elevated levels of unspecified serum peptides in COPD patients as

Figure 4 IDO activity as depicted by Kyn/Trp ratio.

Notes: Tryptophan and kynurenine levels were measured in plasma by SRM. Kyn/Trp values were significantly higher at day 0 compared to day 30 ($P=0.0352$) and higher at day 0 than in controls ($P=0.0338$).

Abbreviations: IDO, indoleamine 2,3-dioxygenase; SRM, selective reaction monitoring; Kyn/Trp, kynurenine/tryptophan.

measured by immunoassays. Using metabolomic profiling, protein degradation products have been detected in the serum of COPD patients, particularly those with emphysema and cachexia.²⁷ We have reported peptides in bronchoalveolar lavage fluid in COPD, many consistent with elastase activity.²⁸ Further studies are necessary to determine if these would serve as a biomarker for AECOPD.

There are several limitations of this study. First, our longitudinal samples were limited to day 30 post-AECOPD. Therefore, we do not know whether tryptophan levels remained low or continued to increase relative to controls. To identify biomarkers of AECOPD, we matched controls for lung function who were also frequent exacerbators, but who had not experienced an exacerbation for at least 1 month. Therefore, we do not know whether frequent exacerbators had different tryptophan levels and catabolism relative to healthy controls or COPD patients who do not experience exacerbations. Therefore, the role of tryptophan catabolism in frequent or prolonged exacerbations warrants future investigation.

Conclusion

Patients with an AECOPD have a unique plasma metabolomic signature at the initiation of their exacerbation. This signature includes an increase in the Kyn/Trp ratio consistent with an increase in IDO activity. The role of tryptophan catabolism during AECOPD warrants further investigation.

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Author contributions

CW, MG, and TG conceptualized the project; MG, CB, BW, SH, and BS carried out experiments; CW, MG, CR, and CB carried out data analysis, CW and MG participated in writing

the manuscript, and TG, PW, CB, BW, and BS critically reviewed the manuscript. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interests in this work.

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Supplementary materials

Table S1 Transitions for tryptophan and kynurenine

Metabolite	Q1 <i>m/z</i>	Q2 <i>m/z</i>
Tryptophan	204.892	188
Tryptophan	204.892	169.9
Tryptophan	204.892	158.96
Tryptophan	204.892	117.908
Kynurenine	208.92	191.904
Kynurenine	208.92	145.943
Kynurenine	208.92	135.957
Kynurenine	208.92	94.049
Tryptophan ¹³ C11	216	199
Tryptophan ¹³ C11	216	169
Tryptophan ¹³ C11	216	154
Tryptophan ¹³ C11	216	140.9
Kynurenine D6	215	198
Kynurenine D6	215	150.9
Kynurenine D6	215	142
Kynurenine D6	215	98.2

Note: Q1 and Q2, first and second mass analyzers.

Abbreviation: *m/z*, mass/charge.

Table S2 Analytes differentially expressed comparing day 0 to day 30

RT	<i>m/z</i>	Putative identification
0.4092	192.0339	
0.3653	227.1242	
1.5818	229.1532	Peptide
0.6904	229.1537	Peptide
0.6979	251.1345	Peptide
3.5796	285.6607	
1.0920	293.0517	
0.6929	319.1215	
1.0963	343.0335	
1.0904	395.0595	
1.0927	401.0747	
1.0978	411.0227	
2.2749	416.2397	
2.2383	436.2067	Peptide
2.2804	438.2229	
1.9731	440.2379	
0.3585	448.668	
1.6195	456.2284	Peptide
2.2801	479.2485	Peptide
6.5000	480.3403	Lipid
6.4761	482.3572	Lipid
6.5022	502.3207	Peptide
6.4766	504.3396	
5.2860	509.3309	
1.0933	531.0327	
13.1409	531.4049	
2.2757	541.2188	Peptide
13.1434	594.4145	Lipid
6.4827	606.3066	Peptide
1.6767	709.0683	
13.1658	940.523	

Abbreviations: RT, retention time; *m/z*, mass/charge.

Table S3 Analytes differentially expressed comparing day 0 to controls

RT	<i>m/z</i>	Putative identification
1.8662	163.1318	
1.6614	171.0983	
1.86	185.1138	
1.5791	191.1498	
0.4092	192.0339	
0.3588	193.1538	
0.4057	198.0943	
3.0522	199.1797	
0.3585	203.0523	
0.5741	204.1223	
9.8983	208.0385	
1.6615	212.1249	
2.5382	213.1435	
0.3442	219.026	
3.0513	221.1605	
3.4716	223.0953	
1.8588	226.1407	
1.1927	229.1528	
1.5818	229.1532	Peptide
0.6904	229.1537	Peptide
1.0901	230.0336	
1.5848	240.1584	
13.7318	243.968	
0.3551	244.0788	
3.0993	244.1537	
3.4708	245.078	
1.0961	246.0052	
0.7777	246.0236	
1.6071	246.1656	
8.6263	247.1305	Peptide
3.0976	249.1076	
0.6979	251.1345	Peptide
1.5881	251.1353	
13.7623	251.3842	
1.0976	253.0267	
2.5361	254.1701	
1.6966	255.1202	
0.4398	256.0572	
1.6824	257.0866	
8.8146	259.6639	
0.3524	263.0841	
0.8497	267.0591	
3.8807	269.1376	
0.3485	271.0384	
1.6376	274.0912	
1.0971	276.0376	
0.3572	276.9842	
3.2302	281.1351	
5.1828	283.152	
6.6211	283.2227	
3.475	286.1041	
0.3485	287.0129	
1.0906	287.0312	
2.2739	289.1319	
3.0952	290.1338	Peptide
1.7269	292.0288	

(Continued)

Table S3 (Continued)

RT	m/z	Putative identification
1.6814	292.0944	
1.092	293.0517	
2.0077	295.1871	
1.6749	297.0732	
5.7355	300.1562	Peptide
6.6352	305.2672	
1.0892	309.0231	
2.2719	309.6462	
1.0864	315.036	
4.693	316.1872	Peptide
1.6716	319.0568	
0.6929	319.1215	
1.6643	319.2067	
3.2243	322.1631	
6.6409	322.2928	
7.4187	322.6846	
6.6068	324.2496	
6.6439	327.2493	
0.3577	330.7515	
7.4216	331.6843	
0.3551	332.7515	
1.0892	337.0185	
4.1134	338.2654	Sphingosine
0.6137	339.0849	
1.0963	343.0335	
3.5422	343.2918	
0.3486	344.9705	
7.4247	345.688	
1.6143	346.0417	
5.1858	346.1605	Peptide
1.1105	346.9641	
0.3591	349.1201	Peptide
6.6633	349.2919	
13.0941	352.2866	
3.8858	354.127	Peptide
0.3356	355.0009	
7.9032	355.2823	
2.269	357.2031	Lipid
8.8104	357.2973	
1.5944	363.05	
3.5481	365.2758	
6.6604	366.3192	
1.72	367.9704	
0.3604	371.1011	
6.6651	371.2745	
4.726	371.3242	
13.1143	372.7997	
1.0931	373.074	
7.4528	376.3162	
7.901	377.2642	
8.8109	379.2798	
1.6829	381.0287	
0.3593	383.114	
1.6636	387.1926	
2.1815	389.1957	
0.3536	390.7096	
4.7366	393.3058	

(Continued)

Table S3 (Continued)

RT	m/z	Putative identification
1.0904	395.0595	
1.6779	395.0655	
0.3563	399.0878	
6.2208	400.34	
1.0927	401.0747	
1.9865	402.2247	
0.3465	402.9335	
2.0137	404.2393	
1.09	405	
0.3504	405.096	
1.6048	410.0573	
6.6635	410.3434	
1.0978	411.0227	
0.9505	411.1445	
10.2998	411.2636	Peptide
2.2901	413.1735	Peptide
6.6685	415.2991	
2.2749	416.2397	
1.9486	418.2552	Peptide
1.0939	420.9697	Myo-inositol
0.4032	421.0134	
13.056	422.1534	Peptide
1.986	424.2069	Peptide
2.0219	426.2226	Peptide
3.3009	430.3147	
3.8793	435.1404	Peptide
2.2383	436.2067	Peptide
1.9411	438.2215	
2.2804	438.2229	
7.9047	439.2336	Peptide
1.9731	440.2379	Peptide
0.3602	441.0728	
8.8391	441.2505	
0.361	443.07	Peptide
1.6293	443.2068	Peptide
7.9101	445.253	Peptide
1.6022	446.0764	
0.3583	446.6695	
0.4907	447.1107	
0.3585	448.668	
1.6604	450.0504	
0.352	451.1009	
13.1007	451.3301	
2.1912	454.2176	
6.6619	454.3717	
6.6553	455.4541	
0.571	456.0049	
1.6195	456.2284	Peptide
6.67	459.3263	Peptide
1.7063	459.9791	
0.4394	463.0131	
0.7459	463.0142	
1.6884	463.0538	
1.5937	464.0727	Peptide
0.3345	464.9774	
1.9822	465.2359	Peptide
12.737	467.0998	

(Continued)

Table S3 (Continued)

RT	m/z	Putative identification
3.473	467.1661	Peptide
10.3003	467.3253	
4.9923	468.3055	
0.987	469.0545	
13.1096	469.3385	
1.7225	470.9654	
1.6195	472.0268	
1.0919	479.012	
1.6744	479.1225	Peptide
2.2801	479.2485	Peptide
8.8117	482.2728	Peptide
5.5895	482.3202	
7.9175	485.1119	
4.993	490.2884	Peptide
6.1727	496.3386	Lipid
6.6598	498.3931	
0.3607	499.0317	
0.3661	501.0279	
5.5152	502.2903	Peptide
6.5022	502.3207	Peptide
6.6618	503.3524	
5.588	504.3023	Peptide
9.342	505.1728	Peptide
7.906	507.2218	Peptide
0.3502	509.0611	
8.8148	509.2378	Peptide
6.6027	510.3528	Lipid
0.4088	511.1077	
10.3056	512.3832	
1.6796	514.1279	Peptide
4.3737	514.3133	Peptide
0.3597	515.0057	
0.374	517.1219	
6.1755	518.3203	Lipid
0.3419	518.8442	
5.7313	520.3393	
10.2989	521.3353	Peptide
6.4842	522.3544	Lipid
6.6752	522.355	
7.4224	524.37	Lipid
1.9592	527.2105	Peptide
10.3044	528.3798	Peptide
5.902	530.3202	
10.3126	530.3374	Peptide
0.3349	530.8702	
1.0933	531.0327	
0.3529	532.9185	
13.773	536.1627	
10.7768	537.3705	
0.3335	538.905	
0.3693	539.1055	
5.7297	539.3104	
13.7723	541.1272	Peptide
2.2757	541.2188	Peptide
5.7333	542.3218	
6.6533	542.4236	
1.952	543.2365	Peptide

(Continued)

Table S3 (Continued)

RT	m/z	Putative identification
2.2779	546.1989	Peptide
7.4211	546.3526	Lipid
6.6677	547.3805	
0.4231	548.0531	
9.2218	554.1747	Peptide
0.3499	554.8997	
1.6083	555.0536	
0.37	555.0773	
2.2743	557.1907	
9.2188	559.1309	
0.3603	560.9874	
7.4221	562.328	Peptide
13.6	563.393	
2.2754	568.1795	Peptide
5.5875	572.2921	Peptide
1.5933	572.3243	
5.7311	573.3019	Peptide
1.6231	574.0328	
1.6091	577.0354	
7.4223	577.3347	Peptide
13.1318	577.4431	
1.6266	585.0671	
2.606	585.2705	Peptide
6.6434	586.4511	
10.3024	590.4088	
6.932	590.4251	
6.6434	591.4084	
6.4549	592.2653	Peptide
1.6239	598.3267	
6.8086	600.3238	Peptide
1.6056	601.1917	
1.7112	603.939	
5.7322	604.2916	
6.4827	606.3066	Peptide
0.3469	607.0939	
7.415	608.3224	Peptide
5.392	611.2865	Peptide
6.6809	612.3241	Peptide
7.4196	614.3404	
0.3622	619.0479	
4.3825	620.3067	Peptide
0.3506	626.9787	
13.6773	627.453	
2.2746	630.1508	
6.6362	630.4782	
0.5446	633.0673	
10.5995	633.1485	
3.4554	633.2536	Peptide
0.3616	635.0202	
13.6383	635.3657	
6.6358	635.4317	
10.725	637.4437	
1.6034	639.0026	
0.359	644.7984	
7.4254	646.2822	
1.6088	649.9961	
0.3528	655.0219	

(Continued)

Table S3 (Continued)

RT	m/z	Putative identification
5.7638	665.2701	Peptide
0.3547	670.9943	
1.7155	673.9541	
6.6237	674.5011	
7.4111	676.3079	
0.3492	676.9997	
12.0071	677.554	
0.3161	678.6746	
6.6254	679.4573	Lipid
1.7114	679.9699	
0.3429	688.7822	
1.7173	689.9271	
0.3507	692.97	
0.3477	694.9721	
13.6232	703.4517	Lipid
13.1358	703.5737	
1.6767	709.0683	
11.7477	712.5443	
7.4314	713.3025	
9.9864	717.632	Lipid
6.6167	718.5284	
13.4943	722.551	Ceramide
6.6094	723.4853	
5.7355	725.4297	Lipid
13.1381	725.5549	
7.4214	729.4603	
13.5057	729.5885	
0.3477	734.953	
1.6269	735.4944	Lipid
11.4089	739.5345	
13.8137	740.5197	Lipid
10.8899	741.5508	
1.9897	743.3704	
7.4197	744.2956	
13.493	751.567	Lipid
13.1615	754.0566	
10.4529	758.5868	
0.5736	759.0603	
7.7516	760.5809	Lipid
13.0793	764.0389	
13.1581	765.0463	
6.5956	767.509	
2.2768	771.4027	
13.132	771.5631	
0.3524	772.9186	
12.8808	774.5722	

(Continued)

Table S3 (Continued)

RT	m/z	Putative identification
12.293	778.5329	Lipid
13.1639	782.5684	Lipid
13.1358	793.5442	
10.3346	794.5649	Lipid
13.7571	796.561	
7.4286	797.4589	
1.599	798.3899	
5.7361	798.9721	
5.7311	801.9803	
13.1657	804.5514	Lipid
14.361	806.5633	Lipid
12.9319	806.5706	Lipid/ceramide
13.2426	810.6704	
0.3509	810.8878	
2.2829	812.4288	
8.6836	820.4116	
12.6246	820.5522	Lipid
13.0707	827.5999	
12.9375	828.5527	Lipid
13.9946	830.568	Lipid
6.1864	830.961	
5.7348	835.9755	
0.3123	836.6608	
6.4852	839.497	
1.7063	841.9379	
7.4234	842.023	
10.3171	847.4601	
11.7131	856.5728	
5.7373	866.9606	
5.7347	869.9697	
13.1642	872.5377	Lipid
8.8102	876.5694	
11.9	882.5886	
13.0421	884.6029	
12.9953	893.0155	
12.9321	896.5377	
5.7506	900.2364	
5.7392	900.9573	
7.4281	910.0114	
13.1352	929.5189	Lipid
11.7452	939.4679	
13.1658	940.523	
1.6171	954.6025	
11.4692	955.5848	
13.0992	963.574	
3.5652	979.9296	

Abbreviations: m/z, mass/charge; RT, retention time.

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