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Lipidome signatures of metastasis in a transgenic mouse model of sonic hedgehog medulloblastoma

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Abstract

Medulloblastoma (MB), the most common malignant pediatric brain tumor, has high propensity to metastasize. Currently, the standard treatment for MB patients includes radiation therapy administered to the entire brain and spine for the purpose of treating or preventing against metastasis. Due to this aggressive treatment, the majority of long-term survivors will be left with permanent and debilitating neurocognitive impairment, for the 30–40% patients that fail to respond to treatment, all will relapse with terminal metastatic disease. An understanding of the underlying biology that drives MB metastasis is lacking, and is critically needed in order to develop targeted therapeutics for its prevention. To examine the metastatic biology of sonic hedgehog (SHH) MB, the human MB subgroup with the worst clinical outcome in children, we first generated a robust SmoA1-Math-GFP mouse model that reliably reproduces human SHH MB whereby metastases can be visualized under fluorescence microscopy. Lipidome alterations associated with metastasis were then investigated by applying ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) under positive ionization mode to primary tumor samples collected from mice without ($n = 18$) and with ($n = 7$) metastasis. Thirty-four discriminant lipids associated with SHH MB metastasis were successfully annotated, including ceramides (Cers), sphingomyelins (SMs), triacylglycerols (TGs), diacylglycerols (DGs), phosphatidylcholines (PCs), and phosphatidic acids (PAs). This study provides deeper insights into

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dysregulations of lipid metabolism associated with SHH MB metastatic progression, and thus serves as a guide toward novel targeted therapies.

Keywords

Medulloblastoma; Metastasis; Non-targeted metabolomics; Ultra-performance liquid chromatography-mass spectrometry; Lipids

Introduction

Medulloblastoma (MB) is an aggressive embryonal tumor that originates in the posterior fossa and frequently metastasizes throughout the central nervous system (CNS). It is the most common malignant pediatric brain tumor, accounting for about 20% of childhood cases [1, 2]. Currently, the standard treatment for all MB patients consists of tumor surgical excision, chemotherapy, and radiation therapy administered to the entire brain and spine for the explicit purpose of treating or preventing against metastasis [3]. With this treatment, the overall 5-year survival rate for pediatric MB patients has reached 73% [4, 5]. However, the vast majority of survivors will suffer permanent and debilitating neurocognitive impairment as a result of the radiation treatment received [2, 6, 7]. For the remaining one-third of patients who fail to respond to standard radiation and chemotherapy, all will relapse with terminal metastatic disease and die within 5 years following diagnosis [1]. To date, an understanding of the underlying biology that drives MB metastasis is lacking, despite a wealth of information from large-scale genomic analyses of human MB. Therefore, there is great clinical need to identify robust biomarkers predictive of risk for metastasis in order to better tailor therapy to individual patients with different risk status [8-10], and to understand the mechanisms promoting MB metastases, which will eventually lead to more effective and less toxic targeted therapies.

MB is comprised of four molecular subgroups: wingless (WNT), sonic hedgehog (SHH), Group 3, and Group 4 [5, 11, 12]. The WNT and SHH subgroups were named for the signaling pathways involved in the pathogenesis of the tumor, with less known about the underlying biology of the remaining two subgroups. SHH MB accounts for one-third of all MB cases [13] and tends to occur in very young children, a population especially vulnerable to the adverse effects of radiation, whereas older children with SHH MB have a relatively poor prognosis [14]. Moreover, metastatic disease is observed in approximately 30% SHH MB, and constitutes the highest risk group (~50% survival) [15]. To investigate the mechanisms of SHH MB metastasis, we used a robust genetically engineered mouse model (GEMM) of SHH MB that was developed by activating SmoA1 in mouse cerebellar granule neuron precursors [16]. The SmoA1 GEMM faithfully recapitulates human SHH MB, with a tumor incidence of ~94%, and metastases observed in ~30% of mice [17]. To more easily visualize metastases, we generated a SmoA1-GFP mouse model by crossing SmoA1 mice with Math1-driven GFP reporter mice, whereby GFP-positive tumors can readily be observed in the brain and spine under fluorescence microscopy [18].

Recent studies have investigated metastasis drivers in both mouse and human SHH MB at the molecular level. The RAS/MAPK signaling pathway [19, 20], Arnt and Gdi2 genes [21],

the protease Usp7 [22], and the NOTCH pathway transcription factor ATOH1 [23] have all been associated with the promotion of SHH MB metastasis. These alterations in signaling pathways and genetic mutations likely affect the tumor microenvironment, which in turn is known to have a profound effect on tumor metabolism [24]. Research on metabolic alterations associated with SHH MB metastasis, however, has been limited to date.

Metabolomics is a powerful tool for cancer research, especially for uncovering metabolic phenotypes useful in diagnosis, prognosis, and therapeutics [25]. Compared with genomics and proteomics, metabolomics provides a more instantaneous view of the status of the biological system, readily reflected in biofluids and tissues [26]. In a recent study, we investigated metabolic alterations in metastasizing and non-metastasizing primary tumors on whole brains from a SHH MB mouse model using three-dimensional matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI). Results revealing alterations in phosphatidic acids (PAs), phosphatidylethanolamines (PEs), phosphatidylserines (PSs), and phosphatidylinositols (PIs) were associated with MB metastasis [27]. Due to the high concentration of lipids in the CNS, and their crucial roles in cell signaling, altered lipid profiles are highly informative of brain disease states [28].

In this study, MB primary tumors from the SmoA1-Math1-GFP GEMM, with and without GFP-positive metastases, were profiled following a non-targeted ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS) lipidomics strategy. Compared with MALDI-MSI, UPLC-MS enables a more comprehensive lipid coverage, but at the cost of spatial information [29, 30]. Thirty-five differentially expressed lipids were identified that differed between non-metastatic and metastatic SHH MB primary tumors, indicating their association with SHH MB metastasis. This investigation provides the first metabolic signatures of SHH MB metastasis, a key step toward developing clinically relevant biomarkers and preventative treatments.

Materials and methods

Chemicals

LC-MS grade acetonitrile, water, 2-propanol, ammonium formate, and formic acid (99.5+%) were purchased from Fisher Chemical (Fisher Scientific International, Inc. Pittsburgh, PA) and used to prepare mobile phases.

Animals

Transgenic SmoA1-Math1-GFP mice were used for this study. These SmoA1-Math1-GFP mice were generated by crossing SmoA1 mice with Math1-driven GFP reporter mice. All mice typically developed detectable MB tumors by magnetic resonance imaging and clinical symptoms at approximately 23 weeks. Mice information can be found in Table S1 of the Electronic Supplementary Material (ESM). Mice brain and spinal cord were dissected from each mouse and fluorescence microscopy was used to visualize the tumors. Mice only having a GFP signal in the cerebellum were designated as having a primary tumor without metastasis (PT group, $n = 18$). Mice having a strong GFP signal in the cerebellum and positive GFP signal in the spinal cord were designated as having a primary tumor with

metastasis (PTMT group, $n = 7$). All animal experiments were performed under protocols approved by Emory University and were in compliance with state and federal Animal Welfare Acts.

Sample collection and preparation

With aid of GFP visualization, the primary tumor in each mouse brain was cut from the cerebellum, immediately frozen using liquid nitrogen, and stored at -80°C . Frozen mice brain tissue samples were cryo-pulverized with liquid nitrogen and weighed. 2-Propanol ($20\ \mu\text{L}\ \text{mg}^{-1}$) was added to the cryo-pulverized tissue samples to precipitate proteins. Samples were vortex-mixed for 1 min and centrifuged at 13,000 rpm for 7 min. Following centrifugation, $100\ \mu\text{L}$ of supernatant was transferred to an autosampler vial and analyzed by UPLC-MS. A blank sample, prepared with $100\ \mu\text{L}$ 2-propanol, underwent the same process as the murine tumor samples. A pooled quality control (QC) sample was prepared by mixing a $10\ \mu\text{L}$ aliquot of supernatant of each sample and was analyzed every 8 runs to assess UPLC-MS system stability. All samples were run in randomized order on consecutive days.

UPLC-MS analysis

UPLC-MS analyses were performed using a Ultimate 3000 UPLC (Thermo Fisher Scientific, Inc., Waltham, MA) system coupled to a Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA). Reverse-phase chromatography was performed with a Waters ACQUITY UPLC BEH C18, $2.1 \times 50\ \text{mm}$, $1.7\ \mu\text{m}$ particle column. The mobile phases and chromatographic gradients used were described in our previous study [31]. The mass spectrometer was operated in both positive and negative ion modes. All chromatographic and MS parameters can be found in the ESM (Tables S2-S3). Representative UPLC-MS data is provided in Fig. S1 (ESM). Although tentative metabolite identities could be assigned to selected features in negative ion mode, tandem MS experiments for this dataset was generally not successful due to the low abundance of the specific features selected by the multivariate models. Therefore, only the positive ion mode dataset was pursued going forward. Data analysis results for the negative ion mode data are still provided in the ESM section for information purposes only. Data-dependent acquisition (DDA) experiments were used to collect MS/MS spectra at stepped normalized collision energy (NCE) of 10, 30, and 50. For compounds missing MS/MS collection by DDA, parallel reaction monitoring experiments were performed at collision energies ranging from 10 to 40 eV to obtain fragmentation data.

Data analysis

Spectral features (retention time (Rt), m/z pairs) were extracted from UPLC-MS data using Progenesis QI v2.1 (Nonlinear Dynamics, Waters Corp.). This procedure included chromatographic peak retention time alignment, peak picking, isotope grouping, adduct analysis, integration, and normalization. After feature extraction, missing value filtering was performed by first removing features that were not present in at least 90% of the samples in one of the PT or PTMT groups, and then, features not present in 80% of all samples were also removed. Data were then $\log_2$ transformed and quantile normalized to generate a symmetrically normal distribution and reduce variation of non-biological origin [32, 33]. Features were selected using variable importance for projection (VIP) scores from partial

least squares discriminant analysis (PLS-DA) [34] and $\log_2$ fold change. VIP scores greater than 2 and $\log_2$ fold change greater than α 1 were used as thresholds. When examining the selected discriminatory feature panel, we observed that 9 of the 47 selected features were ^{13}C isotopic peaks that the Progenesis de-isotoping algorithm had failed to accurately assign. Those ^{13}C isotopic peaks were replaced with their corresponding M (^{12}C) monoisotopic peaks in building multivariate models. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (oPLS-DA) [35] were performed to assess the discriminating power of metabolite datasets in unsupervised and supervised manners, respectively. Data were preprocessed by autoscaling prior to oPLS-DA and PCA, and cross-validated using the random sample subset approach with 10 iterations.

Metabolite identification

Selected discriminant features were subjected to metabolite identification. Elemental formulas were first generated using exact masses measured with a maximum mass error of 5 mDa and isotopic patterns using Progenesis QI v2.1. The tentative identities were then searched against publicly available databases, including the Human Metabolome Database (HMDB) [36], Lipid Maps [37], and Metlin [38]. Both the accurate mass measurements and generated elemental formulas with a mass error of 5 mDa were used to determine possible endogenous metabolite candidates. The identities of possible candidates were further confirmed by matching MS/MS fragmentation patterns to entries in the HMDB, Lipid Maps, Metlin, and literature searches.

Results and discussion

A total of 12,267 spectral features were extracted from the UPLC-MS positive mode dataset. After missing value filtering, 8436 features were retained. Based on PLS-DA VIP and fold change values, 47 features were selected as a best first set to differentiate between PT and PTMT (Fig. 1), 40 out of these 47 features had a p value lower than 0.05 in Student's t test between PT and PTMT classes (Table 1). The relatively small number of discriminant features selected for the final model could be attributed to the relatively small differences observed between primary tumors leading to metastatic or non-metastatic MBs. The selected features were subject to the metabolite annotation pipeline using high-resolution MS/MS experiments, following methods described above.

Multivariate classification performance

To investigate differences in metastasized and non-metastasized MB primary tumor samples at the lipidome level, the set of 8436 filtered features was first utilized to build an exploratory oPLS-DA model. Performance characteristics of this first model (Fig. S2 in ESM) were relatively poor, with 61.1, 7.1, and 34.1% cross-validated sensitivity, specificity, and accuracy, respectively. Seventeen samples were systematically misclassified. The low sensitivity and classification accuracy observed indicated a high degree of biological similarity between the primary tumor samples in the two groups at the lipidome level. This model employed a total of three latent variables and interpreted 53.0% and 33.6% variance from the X (feature peak area) and Y (sample class membership) blocks, respectively. We further evaluated the discriminant power of a second oPLS-DA model built with only 47

features (Fig. 2a, b). These features were selected based on their VIP scores, as described under “Materials and methods.” Performance characteristics of this model were 87.8, 68.6, and 78.2% for the cross-validated sensitivity, specificity, and accuracy, respectively, a significant improvement over the first model using all variables. In this second PLS-DA model, five samples were systematically misclassified, including two PT samples and three PTMT samples. PT mice labeled as SG218 and SG204 were misclassified as PTMT mice. This misclassification could be possibly explained if these mice had developed a small amount of metastasis at the time of tissue collection, but the GFP signal was too weak to be detected so that the mice were incorrectly assigned to the PT group. Three PTMT mice labeled SGTA13, SGTA12, and CASE41 were misclassified as PT mice. This misclassification was difficult to explain and, despite all efforts in examining the specific characteristics of the involved mice, resisted any plausible explanation.

The second oPLS-DA model described above employed two latent variables and interpreted 44.7% and 31.6% variance from the *X* and *Y* blocks, respectively. Although this model’s performance was far from perfect, it was still acceptable as it captured many of the molecular differences between the PT and PTMT groups, leading to interesting biological findings. PCA was also utilized to assess the discriminant performance of the initial 8436-feature set and the selected 47-feature set in an unsupervised manner. Score plots revealed better separation between PT and PTMT groups using the 47-feature set (Fig. S3 in ESM). We observed three PTMT samples (SGTA13, SGTA12, and CASE41) overlapped with the PT sample cluster, which was consistent with the oPLS-DA classification model performance. As the brain tumor samples were collected from both male and female mice, to exclude the sex factor, we further developed an oPLS-DA model using only the female mice samples (14 PT and 5 PTMT female mice). The performance of this new model (Fig. S4 in ESM) was still very close to the prior model, with 88.7, 66.0, and 77.3% as cross-validated sensitivity, specificity, and accuracy, respectively. Three samples were misclassified, including two PT samples (SG218 and SG204) and one PTMT sample (SGTA12), which was consistent with the model using all mice samples. Therefore, we found no observed correlation between sex and the lipid differences of the selected features between the groups. Compared with the performance of the model using the complete feature set, the specificity and classification accuracy of the models using selected features were greatly improved and provided the basis for an examination of the biological differences associated with MB metastasis.

Discriminant metabolite annotation

Out of the 47 features in the best oPLS-DA model, 16 features were increased in the PT group, while the remaining 31 features were increased in the PTMT group. A total of 34 features were successfully annotated with various levels of confidence by both high-resolution MS and MS/MS (Table 1). Table S4 (ESM) provides detailed MS/MS fragmentation data and annotation confidence levels for each of the identified species. These were assigned mostly to lipid species, including glycerolipids (GLs), glycerophospholipids (GPLs), and sphingolipids (SPs). Other small molecules, including peptide and steroid hormones, were also identified.

Metabolic alterations associated with MB metastasis

Twenty of the identified features were GLs, including triacylglycerols (TGs) and diacylglycerols (DGs). GLs are comprised mainly of mono-, di-, and tri-substituted glycerols, the fatty acid esters of glycerol. TGs are the major metabolic energy storage molecules in living organisms. Synthesis and storage of TGs have important roles in metabolic homeostasis, with two main TG synthesis pathways: the glycerol-3-phosphate and the monoacylglycerol pathways [39]. In most mammalian cells, the glycerol-3-phosphate pathway is primarily utilized for de novo TG synthesis [40] (Fig. 3). The first step is catalyzed by glycerol-3-phosphate acyltransferases (GPATs), which occurs in the endoplasmic reticulum (ER) and mitochondria [41, 42]. A fatty acid is then transferred to lysophosphatidic acids (LPAs) by 1-acylglycerol-3-phosphate acyltransferases (AGPATs) to produce PAs [43]. PAs are then converted to DGs through the enzymatic activity of phosphatidate phosphatases (PAPs). The activity of PAP1 is determined by the lipin family of proteins, including lipin-1, lipin-2, and lipin-3 [44]. As the immediate precursor of TGs, DGs are catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes to produce TGs in the final step [45]. In the alternative monoacylglycerol pathway, MGs are targeted by acyl-CoA:monoacylglycerol acyltransferase (MGAT) enzymes to generate DGs, followed with DGAT enzymatic activities to generate TGs [40]. In mammals, this pathway mainly occurs in intestinal enterocytes [46, 47]. In this study, we observed increased levels of TGs in the PTMT group (Table 1), suggesting an association with MB metastasis. As TGs provide a reservoir of fatty acids that can be mobilized for energy generation, the elevation of TG abundances in the PTMT group may be attributed to an increased energy need for MB metastasis. In agreement with these findings are reports that cancer cells contain a higher abundance of lipid droplets as the major form of TG storage in tumor tissues [48]. Higher levels of polyunsaturated fatty acids are also found to promote brain cancer metastasis through peroxisome proliferator-activated receptor γ (PPAR γ) signaling [49]. DGs are important intermediates in lipid metabolism and act as second messengers in cell signaling [50]. Two DG species were detected as increased in the PTMT group, and one in the PT group. This alternating trend could be related to the downstream synthesis to the following TGs and GPLs. For example, an increase of DG(18:1_18:2) was observed in the PTMT group, which was also accompanied by a corresponding increase of related TGs with the (18:1, 18:2) fatty acid chains in this group. The increase of DG(32:2) in the PT group could be related to the corresponding increase of GPLs observed in the same group. Among the identified fatty acid chain compositions of GLs, the unsaturated form of stearic acids C18:1 and C18:2, and palmitic acid C16:0 and its unsaturated form 16:1 have been frequently observed as being increased in PTMT group. In agreement with our findings, higher levels of those fatty acid chains were observed in human glioma tumors compared with nonmalignant brain tissues [51]. The altered fatty acid composition of GLs is regulated by GPATs [52]. However, the biological role of lipids in the brain tumor metastasis at the acyl chain level is currently poorly understood.

Three of the identified features were GPLs. As the key components of cellular membrane, GPLs play important roles in multiple cellular processes, including energy storage, cellular signaling, and cell-to-cell interactions [53]. Because such cellular activities are associated with cell transformation, cancer progression, and metastasis, emerging evidence has

suggested that GPLs could be promising biomarkers for various cancers [54-56]. In mammalian cells, GPLs are also synthesized through the glycerol-3-phosphate pathway. As branching points, PAs and DGs serve as precursors of GPLs in that pathway [43, 57] (Fig. 3). Along these lines, we observed increased levels of GPLs in the PT group, including phosphatidylcholines (PC(32:1(OH)), PC(36:3)), and a phosphatidic acid (PA(40:2)). Elevation of the choline phospholipids PCs has been noted as one of the unique characteristics of cancer cells [58]. In agreement with our findings, increased choline levels have been reported in different types of brain tumors, including MB [59-61]. The decrease of PA(40:2) in the PTMT group can be attributed to the enhanced activity of PAPs in the glycerol-3-phosphate pathway to produce GLs [62]. In line with these findings, PAP inhibition has been reported to counteract cancer cell proliferation [63].

Ten of the identified features were SPs, including sphingomyelins (SMs), ceramides (Cers), and their metabolites, ceramide phosphoethanolamines (PE-Cers). SPs function as structural components of biological membranes and play important roles in cell signaling. They are involved in a variety of cellular processes including differentiation, cellular senescence, apoptosis, and proliferation [64]. Research has identified crucial roles of SP metabolism in cancer development and progression [65]. Ceramides are the central molecules in SP metabolism and function as tumor-suppressor lipids, inducing antiproliferative and apoptotic responses in various cancer cells [65, 66]. Endogenous ceramide levels are regulated through de novo synthesis and/or SM hydrolysis. We observed increased levels of Cers and decreased levels of SMs and PE-Cers in the PTMT group. As ceramides can be formed from SM hydrolysis, or serve as substrates for the synthesis of SM or phosphorylated ceramide metabolites, this alteration could be attributed to sphingolipid-mediated responses against MB metastasis. Sphingosine (d18:1) or sphinganine (d18:0) were constantly observed as the backbone of the identified SPs. The fatty acid compositions of identified Cers were in the range of 20 to 24 carbons in length. The assembly of different lengths of fatty acid chains in Cers is regulated by ceramide synthases (CerSs). The CerSs have been reported to play opposing proapoptotic or prosurvival roles in tumors [67, 68]. It has been reported that the pathophysiological roles of ceramides are chain length dependent in various diseases [69]. In agreement with our findings, increased levels of Cers were observed in breast cancer tissue, with fatty acid chains C16:0, C20:0, C24:0, and C24:1 significantly increased in malignant tumors compared with benign tissue [70].

Overall, the results in this study show significant alterations in the abundance of multiple lipid species, providing important insights into the metabolic signatures accompanying SHH MB metastasis. These results are consistent with previously reported increased lipogenesis in SHH MB in cell cultures [71]. Furthermore, these lipid species are known to regulate the RAS/MAPK signaling pathway [72], which is identified to be associated with the promotion of SHH MB metastasis. Validation of such pathways in human SHH MB could then serve for the development of new biomarkers and targets for novel therapies against metastatic MB.

Conclusion

This study employed a UPLC-MS-based discovery lipidomics approach to study metabolic alterations in SHH MB from non-metastatic and metastatic primary tumors from the SmoA1-Math-GFP transgenic mouse model. A panel of 47 discriminant features was selected that correlated with MB metastasis. Out of these features, 34 were annotated. Many lipids in the glycerol-3-phosphate and sphingolipid metabolism pathways were found to be associated with MB metastatic progression. Since the study was somewhat limited by its relatively small number of animals, a larger sample cohort may be needed in the future to further validate the findings presented here. Despite this limitation, the significant results presented in this study indicate that there are detectable lipidome alterations associated with metastasis, providing important insight into the mechanisms driving MB progression. Ultimately, candidate lipid pathways could be targeted for investigation in human MB such that the data could be clinically translatable with the purpose of developing biomarkers to aid in tailoring treatment to improve patients' long-term quality of life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data generated in this work are available through the NIH Metabolomics Workbench (<http://www.metabolomicsworkbench.org/>) with project ID PR000872 (doi: <https://doi.org/10.21228/M8ZT30>, study ID ST001290).

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Fig. 1.

Volcano plot including the total set of 12,267 spectral features detected in positive mode. Variable importance for projection (VIP) from PLS-DA model and $\log_2$ fold change was represented in the y -axis and x -axis, respectively. Features that met selection criteria are highlighted in color. Red dots represent features with increased abundance in PTMT group; blue dots represent features with increased abundance in PT group

Fig. 2.

a oPLS-DA scores plot depicting sample clusters between PT and PTMT samples using the 47-feature panel. Variance between groups is captured across the x -axis. **b** oPLS-DA cross-validated classification plot between PT and PTMT samples using the 47-feature panel. The x -axis represents sample number, and the y -axis represents the cross-validated predicted scores of the binary oPLS-DA classification model. PT and PTMT samples are represented by green diamonds and red squares, respectively. The threshold for sample classification is represented by the green dashed line. Cross-validated sensitivity, specificity, and accuracy values for the model are 87.8%, 68.6%, and 78.2%, respectively

Fig. 3.

Glycerol-3-phosphate pathway for de novo synthesis of glycerolipids (GLs) and glycerophospholipids (GPLs). GPAT, glycerol-3-phosphate acyltransferase; AGPAT, 1-acylglycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; PA, phosphatidic acid; DG, diacylglycerol; DGAT, diacylglycerol acyltransferase; PAP, phosphatidate phosphatase; TG, triacylglycerol

Table 1

Metabolite annotation for the selected feature panel.

VIP Score	Retention Time (min)	m/z		Elemental Formula	Adduct Type	Mass Error (ppm)	Fold Change	Abundance PT/PTMT	p value	Metabolite Identity
		Experimental	Theoretical							
3.02	0.39	478.3221	--	--	--	--	-3.88	11.48/15.37	0.00136	--
2.90	7.22	723.7339	723.7337	C ₄₆ H ₉₁ NO ₃	[M+NH ₄] ⁺	0.28	-1.33	11.41/12.74	0.00188	Cer(d46:1)
2.53	7.21	624.6290	624.6289	C ₄₀ H ₈₁ NO ₃	[M+H] ⁺	0.16	-1.19	13.82/15.01	0.00554	Cer(d18:0/22:0)
2.78	7.60	672.6271	672.6265	C ₄₂ H ₈₃ NO ₃	[M+Na] ⁺	0.89	-1.89	7.85/9.74	0.00557	Cer(d18:1/24:0)
2.61	3.81	741.5516	741.5517	C ₃₉ H ₇₉ N ₂ O ₇ P	[M+Na] ⁺	-0.13	1.25	12.49/11.24	0.00570	SM(d18:1/16:0(OH))
2.39	7.22	658.6113	658.6109	C ₄₁ H ₈₁ NO ₃	[M+Na] ⁺	0.61	-1.13	12.04/13.17	0.0105	Cer(d18:1/23:0)
2.45	7.97	872.7707	872.7702	C ₅₅ H ₉₉ O ₆	[M+NH ₄] ⁺	0.57	-1.17	17.08/18.25	0.0126	TG(16:1_18:1_18:2)
2.22	7.23	636.6287	636.6289	C ₄₁ H ₈₁ NO ₃	[M+H] ⁺	-0.31	-1.27	11.59/12.86	0.0141	Cer(d18:1/20:3)
2.38	8.08	898.7859	898.7858	C ₅₇ H ₁₀₀ O ₆	[M+NH ₄] ⁺	0.11	-1.04	16.14/17.18	0.0152	TG(18:1_18:2_18:2)
2.69	1.25	608.4736	608.4762	C ₃₁ H ₆₃ N ₂ O ₆ P	[M+NH ₄] ⁺	-4.27	2.23	11.80/9.57	0.0157	PE-Cer(d29:1)
2.54	6.55	191.0711	--	--	--	--	-1.94	5.38/7.33	0.0158	--
2.30	8.06	860.7707	860.7702	C ₅₄ H ₉₈ O ₆	[M+NH ₄] ⁺	0.58	-1.84	12.17/14.00	0.0164	TG(15:0_18:1_18:2) TG(16:0_17:1_18:2)
2.34	8.13	874.7862	874.7858	C ₅₅ H ₁₀₀ O ₆	[M+NH ₄] ⁺	0.46	-1.06	15.81/16.86	0.0180	TG(16:0_18:1_18:2)
2.25	8.22	888.8019	888.8015	C ₅₆ H ₁₀₂ O ₆	[M+NH ₄] ⁺	0.45	-1.50	13.32/14.83	0.0182	TG(17:1_18:1_18:1)
2.25	7.76	792.7081	792.7076	C ₄₉ H ₉₀ O ₆	[M+NH ₄] ⁺	0.63	-1.09	12.34/13.43	0.0182	TG(14:0_16:1_16:1)
2.14	5.76	582.5094	582.5092	C ₃₅ H ₆₄ O ₅	[M+NH ₄] ⁺	0.34	1.05	12.21/11.16	0.0194	DG(32:2)
2.18	8.30	972.8959	972.8954	C ₆₂ H ₁₁₄ O ₆	[M+NH ₄] ⁺	0.51	-2.28	10.10/12.38	0.0204	TG(59:3)
2.66	5.89	795.5312	795.5301	C ₄₃ H ₈₁ O ₈ P	[M+K] ⁺	1.38	1.24	10.77/9.53	0.0207	PA(40:2)
2.23	3.13	1408.9870	--	--	--	--	1.95	11.71/9.76	0.0207	--
2.57	6.55	601.5192	601.5190	C ₃₉ H ₇₀ O ₅	[M+H-H ₂ O] ⁺	0.33	-1.59	8.40/9.99	0.0208	DG(18:1_18:2)
2.25	7.33	1017.8678	1017.8692	C ₆₃ H ₁₁₆ O ₉	[M+H] ⁺	-1.38	1.01	13.10/12.09	0.0214	TG(60:3;O3)
2.35	8.47	1135.9090	1135.9087	C ₆₉ H ₁₂₄ O ₁₀	[M+Na] ⁺	0.26	1.42	10.52/9.11	0.0214	--
2.04	8.46	925.7449	--	--	--	--	3.42	11.00/7.58	0.0235	--
2.07	2.82	1152.7360	1152.7356	C ₅₈ H ₁₀₆ NO ₁₈	[M + 2Na-H] ⁺	0.35	-1.34	9.82/11.16	0.0245	Trihexosylceramide (d40:1)

VIP Score	Retention Time (min)	m/z		Elemental Formula	Adduct Type	Mass Error (ppm)	Fold Change	Abundance PT/PTMT	p value	Metabolite Identity
		Experimental	Theoretical							
2.25	7.96	846.7550	846.7545	C ₅₃ H ₉₆ O ₆	[M+NH ₄] ⁺	0.59	-1.16	13.47/14.64	0.0245	TG(14:0_18:1_18:2) TG(16:0_16:1_18:2) TG(16:1_16:1_18:1)
2.14	2.00	748.5494	748.5487	C ₄₀ H ₇₈ NO ₈ P C ₄₀ H ₇₅ O ₉ P	[M+H] ⁺ [M+NH ₄] ⁺	0.94	1.55	11.61/10.07	0.0256	PC(32:1(OH)) PA(37:2(OH))
2.07	8.22	862.7861	862.7858	C ₅₄ H ₁₀₀ O ₆	[M+NH ₄] ⁺	0.35	-1.54	9.97/11.51	0.0256	TG(15:0_18:1_18:1) TG(16:0_17:1_18:1)
2.17	1.40	372.1009	372.1014	C ₁₂ H ₁₉ N ₃ O ₉	[M+Na] ⁺	-1.34	-1.04	3.63/4.67	0.0266	Asp-Thr-Asp Asp-Ser-Glu
2.33	5.14	279.1605	--	--	--	--	-1.80	6.51/8.30	0.0298	--
2.35	2.43	433.3314	433.3312	C ₂₇ H ₄₄ O ₄ C ₂₇ H ₄₃ O ₃	[M+H] ⁺ [M+H-H ₂ O] ⁺	0.46	3.37	12.23/8.86	0.0299	Trihydroxyvitamin D3 3alpha,12alpha,22S- Trihydroxy-Sbeta- cholestan-26-oic acid
2.05	7.97	956.8648	956.8641	C ₆₁ H ₁₁₀ O ₆	[M+NH ₄] ⁺	0.73	-2.36	9.83/12.20	0.0303	TG(18:0_18:0_22:4)
2.16	7.97	942.8492	942.8484	C ₆₀ H ₁₀₈ O ₆	[M+NH ₄] ⁺	0.85	-1.57	12.97/14.54	0.0317	TG(57:4)
2.03	7.96	851.7106	851.7099	C ₅₃ H ₉₆ O ₆	[M+Na] ⁺	0.82	-1.91	8.65/10.56	0.0323	TG(14:0_18:1_18:2) TG(16:0_16:1_18:2) TG(16:1_16:1_18:1)
2.01	9.38	301.1371	--	--	--	--	-1.12	5.71/6.83	0.0352	--
2.00	7.79	844.7396	844.7389	C ₅₃ H ₉₄ O ₆	[M+NH ₄] ⁺	0.83	-2.12	11.30/13.42	0.0373	TG(16:1_16:1_18:2)
2.03	7.77	818.7239	818.7232	C ₅₁ H ₉₂ O ₆	[M+NH ₄] ⁺	0.85	-1.50	12.58/14.08	0.0400	TG(14:0_16:1_18:2)
2.03	6.65	656.5824	656.5824	C ₃₉ H ₇₄ O ₆	[M+NH ₄] ⁺	0.00	1.07	11.02/9.96	0.0400	TG(8:0_12:0_16:0) TG(10:0_12:0_14:0)
2.22	6.33	209.0818	--	--	--	--	-2.26	3.20/5.45	0.0424	--
2.16	5.45	784.5848	784.5851	C ₄₄ H ₈₂ NO ₈ P	[M+H] ⁺	-0.38	2.22	9.93/7.71	0.0451	PC(36:3)
2.26	6.95	630.5798	630.5796	C ₃₉ H ₇₇ NO ₃	[M+Na] ⁺	0.32	-1.07	8.65/9.73	0.0468	Cer(d18:1/21:0)
2.02	5.22	205.0869	--	--	--	--	-1.32	7.50/8.82	0.0546	--
2.10	5.43	196.2464	--	--	--	--	1.13	8.78/7.64	0.0604	--
2.03	1.15	740.5520	--	--	--	--	2.28	11.26/8.98	0.0747	--
2.03	1.22	652.4997	652.5024	C ₃₃ H ₆₇ N ₂ O ₇ P	[M+NH ₄] ⁺	-4.14	1.93	11.95/10.02	0.0784	PE-Cer(31:1)
2.09	6.35	280.1638	--	--	--	--	-1.58	4.15/5.72	0.0811	--
2.19	6.51	672.5925	672.5925	C ₄₃ H ₇₄ O ₄	[M+NH ₄] ⁺	0.00	-2.32	8.23/10.55	0.0831	DG(O-18:0_22:6)
2.12	1.30	520.4214	--	--	--	--	2.24	8.14/5.90	0.0841	--

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Variable importance for projection (VIP) scores, retention time, experimental exact mass, theoretical mass, predicted elemental formula, adduct type, mass error, fold changes (FC, calculated as the base 2 logarithm of the average abundance ratios), average abundance (after quantile normalization and log₂ transformation), and *p* value of abundances between PT and PTMT groups are included. Fatty acid chain information is noted when MS/MS information was available. Positive FC values indicate increased abundance in PT samples, while negative values indicate higher abundance in PTMT samples. The *p* values were calculated using Student's *t* test. For additional fragmentation information and level of confidence for each annotation, please refer to Table S4 (ESM). *TG*, triacylglycerols; *DG*, diacylglycerols; *Asp*, aspartic acid; *Thr*, threonine; *Ser*, serine; *Glu*, glutamic acid; *Cer*, ceramide; *SM*, sphingomyelin; *PC*, phosphatidylcholine; *PA*, phosphatidic acid; *PE-Cer*, ceramide phosphoethanolamine