

Untargeted Lipidomics Analysis to Discover Lipid Profiles and Biomarkers of Rabbit Acne Model and Reveal Action Mechanism of Isotretinoin

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Background: Acne vulgaris (AV), a chronic inflammatory pilosebaceous disorder, affects 80–90% of teenagers. This study aimed to discover lipid profiles and biomarkers of the rabbit ear acne model, and investigate the mechanism of isotretinoin in treating acne at the lipid level.

Methods: Untargeted lipidomic analysis using ultra-high performance liquid chromatography system (UHPLC) coupled to q-extraction plus was performed to identify skin lipid metabolites in blank control (groups C), model group (group M) and isotretinoin group (group T). Multivariate statistical analysis was used to process the lipidomics data.

Results: A total of 43 lipid classes comprising 6989 lipid species were identified from the mass spectrometry data. The orthogonal partial least squares discriminant analysis (OPLS-DA) model demonstrated significant separation in skin lipidomic profiles between group M and group C. With variable influence on projection (VIP) > 1.0 and P-value < 0.05, 299 significantly different lipid metabolites were identified. These lipid metabolites consisted mainly of ceramides (Cer) (53.85%), phosphatidylethanolamines (PE) (9.03%), phosphatidylcholines (PC) (5.35%), and sphingomyelin (SM) (4.01%). Combining with AUC ≥ 0.9 as the elected criteria, Cer (d18;1_24:0), zymosterol (ZyE) (33:5), Cer (t43:1), ZyE (33:6), ZyE (24:7), and ZyE (35:6) have “high” accuracy. Isotretinoin treatment normalized 25 lipid metabolites in the acne model.

Conclusion: Our findings provide new insights into the role of lipid metabolism in the pathogenesis of acne and the action mechanism of isotretinoin.

Keywords: acne vulgaris, lipidomics, isotretinoin, skin, animal model

Background

Acne vulgaris (AV), a chronic inflammatory pilosebaceous disorder, affects 80–90% of teenagers.¹ Its main clinical manifestations include non-inflammatory (comedones) and inflammatory lesions (papules, pustules, cysts, and nodules).² Severe manifestations of acne can be painful, leading to disfigurement and scarring. They may significantly impact self-esteem and cause psychological distress in some individuals.³ The development of acne is influenced by four major pathogenic factors: excessive sebum secretion, hyperkeratinization of follicular keratinocytes, colonization of *Cutibacterium acnes* (*C. acnes*), and inflammatory events.⁴ The quantitative and qualitative modification of skin lipids has been increasingly recognized as a key component in acne pathogenesis.⁵ Skin lipids play a significant role in acne development by influencing skin condition through various mechanisms, including maintaining biochemical, physical, and microecological functions.⁶

Isotretinoin (13-*cis*-retinoic acid), a synthetic analogue of Vitamin A, is commonly used to treat acne and other dermatological diseases.⁷ It rapidly converts intracellularly to all-*trans* retinoic acid, which then binds to retinoid receptors to exert its effects.⁸ Isotretinoin targets four major factors in acne pathogenesis: normalizing follicular desquamation, reducing sebum secretion, inhibiting the growth of *C. acnes*, and exerting anti-inflammatory properties. Translational evidence suggests that the desired anti-acne effects of isotretinoin are based on isotretinoin-mediated apoptosis.^{9,10} In addition, previous studies have demonstrated that isotretinoin can alter human lipid metabolism by significantly reducing sebum production while increasing blood lipids.¹¹ Six months of oral isotretinoin systemic therapy can lead to a 36% decline in skin sebum values.¹² The sebum-suppressive effect of isotretinoin has been related to sebocyte apoptosis with isotretinoin-mediated p21-induced cell cycle arrest and upregulation of pro-apoptotic transcription factors including forkhead box protein O1 (FoxO1) and FoxO3a.^{13,14} Moreover, Zhang et al confirmed that isotretinoin induces a decreased expression of lipid metabolism associated genes.¹⁵ Furthermore, a recent metabolomics study revealed that five lipids and lipid-like molecules were normalized by isotretinoin treatment in a rabbit ear acne model.¹⁶

Lipids are a class of biomolecules with significant biological roles, including structural components of cell membranes and microdomains, as well as functioning as signaling molecules. Dysregulated lipid metabolism is a common feature in many disease states.¹⁷ Lipidomics, a high-throughput analysis technology, specifically examines all lipid molecules in living organisms, allowing for detailed analysis of individual lipid classes, subclasses, and molecular species.^{18,19} There are two primary approaches in lipidomics analysis: untargeted and targeted. Untargeted lipidomics involves the comprehensive analysis of all detectable lipids in a sample without bias, while targeted lipidomics focuses on the accurate quantification of specific lipid molecules.^{20,21} Recently, lipidomics has been utilized in studying various dermatological conditions such as urticaria,²² eczema,²³ atopic dermatitis,²⁴ psoriasis,²⁵ and melasma.²⁶

In this study, an untargeted lipidomics analysis was conducted using an ultra-high performance liquid chromatography system (UHPLC) coupled with q-extraction plus to examine the alterations in lipid profile before and after treatment with isotretinoin. The main objective was to discover lipid profiles and biomarkers of the rabbit ear acne model and to explore the mechanism of isotretinoin in acne treatment at the lipid level.

Materials and Methods

Reagents and Materials

Columbia Blood Agar Base and Brain Heart Infusion Broth were procured from Hopebio. Acetonitrile was obtained from Merck. Coal tar was purchased from Alfa Aesar. Formic acid (FA), ammonium acetate (NH₄AC), ammonium hydroxide, and ammonium fluoride (NH₄F) were purchased from Sigma Aldrich.

Animals and Bacteria

Thirty New Zealand white rabbits (3 months old, weighing 1.7–2.5 kg, Ganzhou, China) were utilized in the animal experiments. Before the experiment, the rabbits underwent a one-week acclimatization period to minimize stress. The rabbits were housed individually to prevent ear scratching and damage and had free access to a standard diet and water. The room conditions were maintained at a temperature range of 20–26°C, relative humidity between 40%–70%, and a 12-hour light-dark cycle. *C. Acnes* ATCC 6919 was purchased from the Guangdong Microbial Culture Collection Center. Bacteria were cultured on Columbia Blood Agar Base under anaerobic conditions in Brain Heart Infusion Broth at 37°C for 72 hours. Subsequently, the bacteria were suspended in sterile PBS at a concentration of 6×10⁸ CFU/mL for experimental purposes.

Animal Model Establishment and Treatment Procedures

The rabbit ear acne model was established as described previously with slight modifications.²⁷ Thirty New Zealand white rabbits were randomly assigned to three groups (n = 10 in each group): blank control group (group C), model group (group M), and isotretinoin group (group T). The rabbits in group C received no treatment, while 2% coal tar was applied uniformly to the central inner ear of each rabbit in groups M and T at a dosage of 0.5 mL/d for a duration of 14 days. Additionally, 100 µL of bacteria solution was intradermally injected at six points in the inner ear, starting on day 7, and

repeated every other day for a total of 4 times. Group T received 20 mg/kg/d of oral isotretinoin starting from day 1. On day 14, rabbits were executed via air embolization. Prior to sampling, ear thickness was measured. Skin tissue was collected from the lesion and split into two sections: one for standard histopathology was fixed in 4% paraformaldehyde, and one for lipidomic analysis was stored at -80°C . All animal procedures were carried out in compliance with institutional guidelines for the care and use of experimental animals and were approved by the Animal Ethical Committee of The Second Affiliated Hospital of Nanchang University.

Hematoxylin-Eosin (H&E) Staining

The skin sample was fixed in 4% paraformaldehyde, and embedded in paraffin, and tissue sections ($5\text{ }\mu\text{m}$) were prepared following standard procedures. Subsequently, the sections were then stained with H&E and observed under light microscopy for pathological analysis.

Sample Preparation for UHPLC-Q-TOF MS Analysis

Lipids were extracted using the MTBE method. Initially, samples were spiked with internal lipid standards and homogenized with 200 μL of water and 240 μL of methanol. Subsequently, 800 μL of MTBE was added, and the mixture was sonicated for 20 minutes at 4°C , followed by a 30-minute incubation at room temperature. The solution was then centrifuged at 14000g for 15 minutes at 10°C , resulting in the collection of the upper organic solvent layer, which was subsequently dried under nitrogen.

LC-MS/MS Method for Lipid Analysis

Reverse-phase chromatography using a CSH C18 column was employed for LC separation. The lipid extracts were re-dissolved in 200 μL of a solution containing 90% isopropanol and acetonitrile, followed by centrifugation at 14000g for 15 minutes. Subsequently, 3 μL of the sample was injected. Solvent A consisted of acetonitrile and water (6:4, v/v) with 0.1% formic acid and 0.1 mM ammonium formate, while solvent B was a mixture of acetonitrile and isopropanol (1:9, v/v) with 0.1% formic acid and 0.1 mM ammonium formate. The initial mobile phase comprised 30% solvent B at a flow rate of 300 $\mu\text{L}/\text{min}$, which was maintained for 2 minutes before being linearly increased to 100% solvent B over 23 minutes. Subsequently, the system was equilibrated with 5% solvent B for 10 minutes. Mass spectra were acquired using the Q-Exactive Plus in both positive and negative modes. The ESI parameters were optimized and standardized for all measurements, with the source temperature set at 300°C , capillary temperature at 350°C , ion spray voltage at 3000 V, S-Lens RF Level at 50%, and the scan range of the instruments set at m/z 200–1800.

Data Processing and Statistical Analysis

The raw data was processed using LipidSearch software version 4.2 (Thermo ScientificTM) for peak alignment, retention time correction and extraction peak area. Adducts of +H and +NH₄ were selected for positive mode searches, while -H and +HCOO were chosen for negative mode searches. Ion peaks with a value of more than 50% missing from the group were excluded from the data extracted from LipidSearch. Following normalization and integration using the Perato scaling method, the processed data were analyzed using R package (ropls) for orthogonal partial least squares discriminant analysis (OPLS-DA). Identification of significantly different lipids was based on a combination of statistically significant thresholds of variable influence on projection (VIP) values derived from the OPLS-DA model and a two-tailed Student's *t*-test.

Results

Histological analysis

Compared to the normal rabbit ears, the rabbit ears in group M exhibited thicker skin with developed comedones and inflammatory papules ([Supplementary Figure 1](#)). Histological analysis showed a significant thickening of the epidermis, follicular pore obstruction, hyperkeratosis of follicular sebaceous glands, enlargement of sebaceous glands, and dermal inflammatory cell infiltration, indicating the successful establishment of the rabbit ear acne model. Conversely, group

T showed improvements in the thickened epidermis, a reduction in sebaceous gland size, and a decrease in inflammatory cell infiltration (Figure 1).

Different Lipid Profiles Between Group M and Group C

According to the International Lipid Classification and Nomenclature Committee, lipid compounds can be categorized into eight groups. Each group can be further subdivided into various lipid classes based on polarity. Additionally, differences in saturation or carbon chain length allow for further classification into distinct lipid species. In this study, a total of 43 lipid classes comprising 6989 lipid species were identified (Figure 2). The prominent lipid metabolite subclasses included ceramides (Cer) ($n = 1369$), triglycerides (TG) ($n = 1190$), phosphatidylcholines (PC) ($n = 732$), monohexosylceramides (Hex1Cer) ($n = 645$), phosphatidylethanolamines (PE) ($n = 590$), and diglycerides (DG) ($n = 479$).

To illustrate the differences in lipid profiles between group M and group C, an OPLS-DA model was constructed. This model demonstrated significant separation in skin lipidomic profiles between the two groups (Figure 3A). The model's performance was assessed by monitoring the model's R^2 (goodness of fit) and Q^2 (predictive ability) values. Permutation tests ($n = 200$) were conducted on R^2 and Q^2 , confirming the reliability of the OPLS-DA model ($R^2 = 0.4121$, $Q^2 = -0.6909$) (Figure 3B). Based on univariate statistical analysis methods, differential analysis was performed on all lipid metabolites ($FC > 2.0$ or $FC < 0.5$, $p\text{-value} < 0.05$), and a volcano plot was used for visual display (Figure 4).



Figure 1 Hematoxylin eosin staining of skin tissues (magnification, $\times 100$). (A): group C; (B): group M; (C): group T.

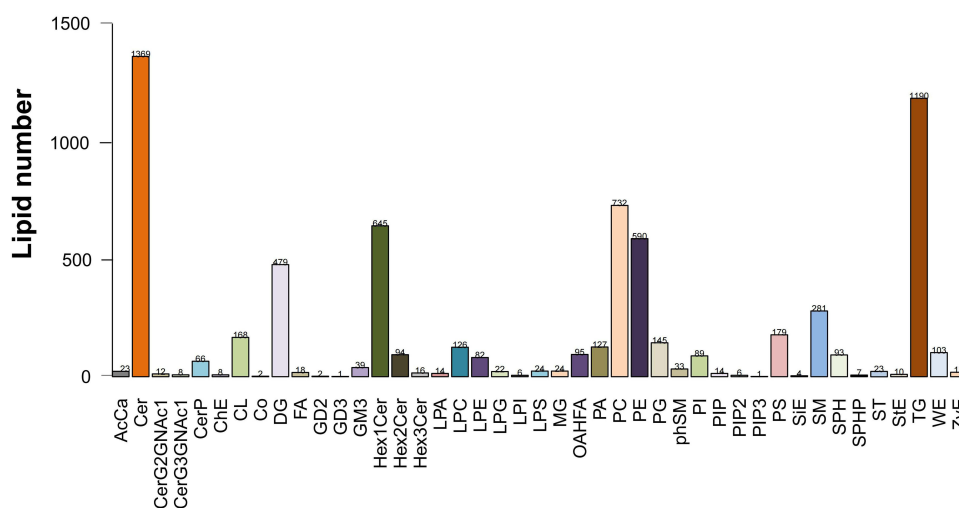


Figure 2 Classification of the identified lipids.

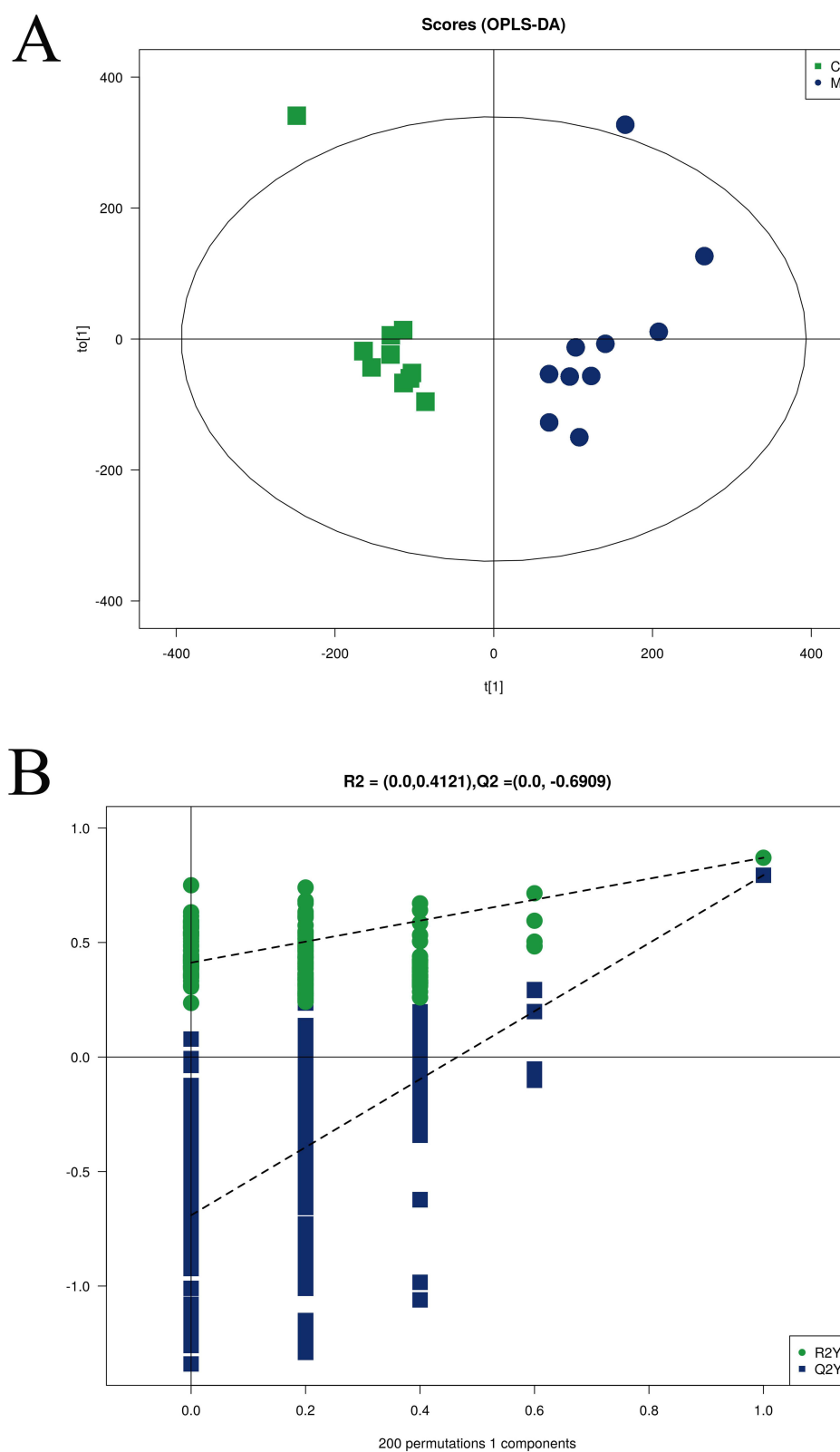


Figure 3 Multivariate statistical analysis of skin lipidomics data. **(A)**Supervised the OPLS-DA model based on all group C and group M samples differentiating the two groups. **(B)** Permutation test for OPLS-DA model.

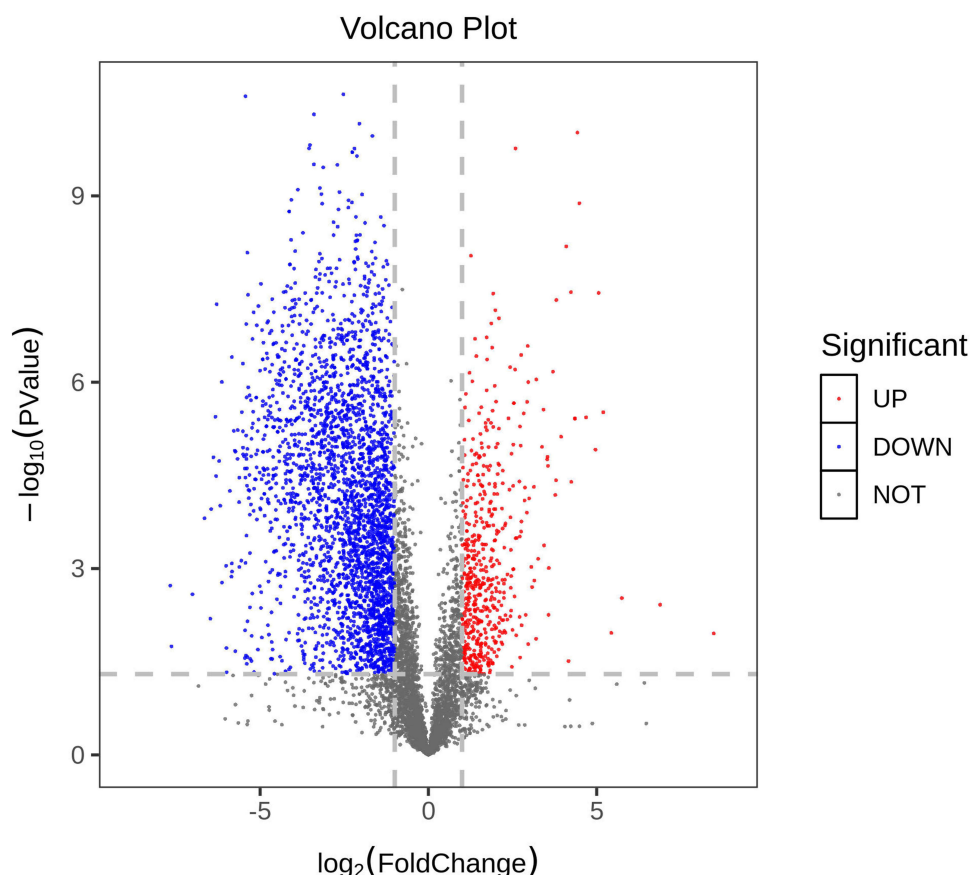


Figure 4 Volcano plots were employed to present the distribution of lipid metabolites from group C and group M, selected by criteria of P-value < 0.05, and fold change > 2.0 or < 0.5.

Screening and Identification of Significantly Altered Lipid Metabolites in the Rabbit Acne Model

A total of 299 differential lipid metabolites were identified between group C and group M in the OPLS-DA models with VIP > 1.0 and $p < 0.05$ ([Supplementary Table S1](#)). In group M, 261 lipid species increased, while 38 lipid species decreased. The chemical classification attribution map highlighted the prominent lipid subclasses as Cer (53.85%), PE (9.03%), PC (5.35%), sphingomyelin (SM) (4.01%), DG (3.01%), phosphatidic acid (PA) (3.01%), lysophosphatidylethanolamine (LPE) (3.01%), zymosterol (ZyE) (3.01%) ([Figure 5](#)). The relative abundance of these differential lipid metabolites was standardized and clustered, and the heatmap illustrated the top 50 differential lipid metabolites between group M and group C ([Figure 6](#)).

ROC Curve Analysis

To assess the predictive value of the screened potential biomarkers, the ROC curves were performed. The ROC curve plots for lipid metabolites with VIP values in the top 10 were shown in [Figure 7](#). AUC values between 0.7 and 0.9 considered to have “moderate” accuracy, and values above 0.9 indicating “high” accuracy. The results demonstrated that Cer (d18:1_24:0), ZyE (33:5), Cer (t43:1), ZyE (33:6), ZyE (24:7), and ZyE (35:6) have “high” accuracy, showing that selected biomarkers could potentially have clinical utility. Among them, Cer (d18:1_24:0) (AUC = 1.000, 95% CI: 1.000–1.000) and Cer (t43:1) (AUC = 1.000, 95% CI: 1.000–1.000) had the highest AUC values, indicating outstanding diagnostic ability for acne.

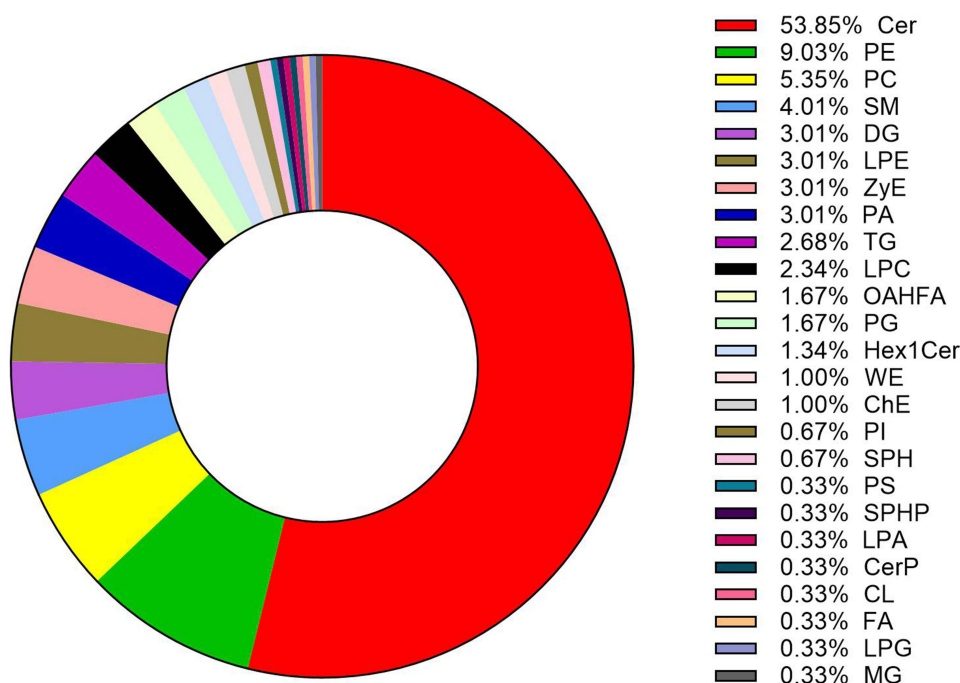


Figure 5 Proportion of identified lipid metabolites in each chemical classification.

Lipid Metabolites Changes After Isotretinoin Treatment

We found that isotretinoin treatment normalized 25 lipid metabolites in the acne model (Table 1). These 25 lipid metabolites included cer (n = 7), SM (n = 5), ZyE (n = 3), PE (n = 2), TG (n = 2), CL (n = 1), LPC (n = 1), PA (n = 1), PC (n = 1), CerP (n = 1), and DG (n = 1). The box plot was employed to show changes of 25 lipid metabolites in the three groups (Figure 8). The heatmap of hierarchical clustering analysis further showed differentially expressed lipids among all the groups (Figure 9).

Discussion

In this study, we investigated the mechanism of isotretinoin in treating the rabbit ear acne model using lipidomics. Our results showed 299 lipid metabolites were markedly altered in group M compared to group C, including Cer, PE, PC, SM, and others. Additionally, isotretinoin was found to normalize 25 of these lipid metabolites.

Significant Differential Lipid Metabolites in the Development of Acne

Ceramides, members of the sphingolipid family, are the building blocks of phospholipid bilayer structure.²⁸ Besides, they play a crucial role in regulating various physiological processes, such as immune regulation, epidermal self-renewal, and preserving epidermal barrier function.²⁹ Previous studies had revealed concentrations of Cer (d42:1), Cer (34:0), and Cer (34:1), ultra-long chain Cers, were significantly increased in a rat model of oleic acid-induced acne.⁵ Consistently, in our study, 161 Cers species were markedly altered in group M, and 158 Cers species were elevated. An abnormal ceramide expression profile is recognized to defect extracellular lipid organization, disturb epidermal self-renewal, and exacerbate skin immune response.³⁰ We hypothesize that the dysregulated levels of ceramides may contribute to inflammatory processes in acne.

In this study, 27 PE species and 16 PC species were markedly altered in the rabbit ear acne model. Notably, the majority of PE (N = 24) and PC (N = 15) species exhibited increased levels. PE and PC are important components of biofilm, belonging to glycerophospholipids.³¹ PE, the second most abundant phospholipid in mammals, plays a multifunctional role in cell processes such as autophagy, membrane fusion, oxidative phosphorylation, and mitochondrial biogenesis.³² Previous study research has shown that PE deficiency in membrane lipids inhibits keratinocyte

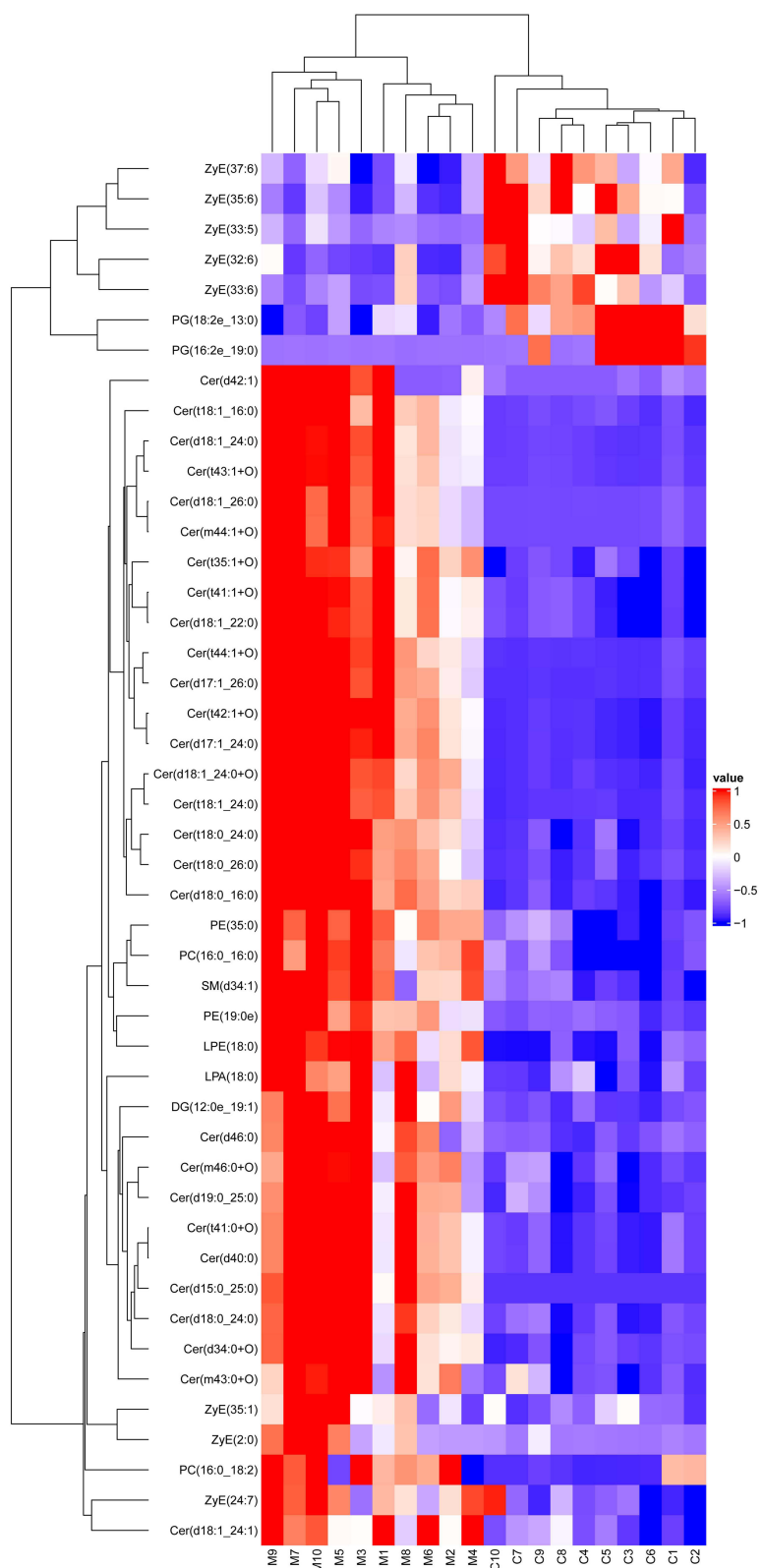


Figure 6 Hierarchical clustering heat map of top 50 differential lipid metabolites between group C and group M. Color scale on the right of the heat map represented normalized values of each identified lipid metabolites content. Red represents significant upregulation, blue represents significant downregulation.

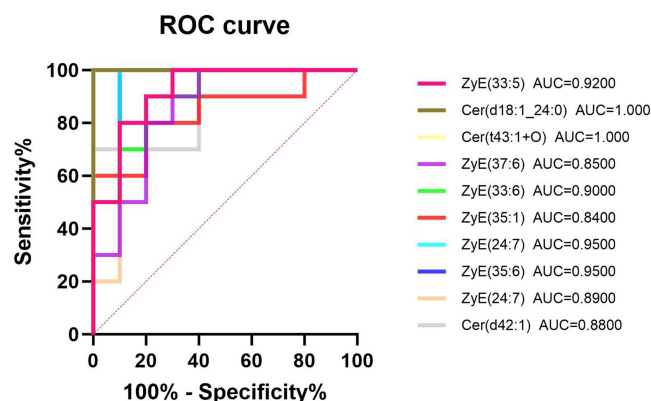


Figure 7 ROC curves for potential lipid biomarkers with VIP values in the top 10.

intercellular networks formation, leading to cell proliferation stops.³³ Hence, altered PE levels observed in our study can prompt the proliferation of keratinocytes, resulting in the thickening of the stratum corneum. Additionally, PC is a primary component of biological membranes in eukaryotic cells, and its degradation and biosynthesis are crucial for

Table I 25 Lipid Metabolites Normalized by Isotretinoin

Name	VIP	C vs M		CalMz	RT-(min)	M ^a	T ^b
		FC	P				
Cer (t41:0+O)	2.7733	0.1819	0.0000	714.6253	14.1725	↑	↓
CL (90:2)-2H	1.0387	0.6884	0.0012	855.6666	11.3600	↑	↓
LPC (16:0)	1.6128	0.0972	0.0107	540.3307	2.8814	↑	↓
PA (20:0_18:1)	1.1270	0.3992	0.0000	729.5440	11.2277	↑	↓
PC (14:1e_18:2)	1.6923	0.4191	0.0000	698.5130	10.1454	↑	↓
PE (16:0_18:1)	1.8513	0.0013	0.0000	716.5236	11.7740	↑	↓
SM (d34:0)	1.7378	0.4009	0.0000	749.5814	9.2599	↑	↓
SM (d42:0)	1.4625	0.2195	0.0000	861.7066	13.8613	↑	↓
SM (d42:2)	1.7733	0.7662	0.0393	857.6753	12.3379	↑	↓
SM (d42:3)	1.5645	0.6425	0.0007	855.6597	11.3711	↑	↓
Cer (d20:0_18:0)	1.8861	0.2958	0.0000	640.5885	13.0760	↑	↓
Cer (d14:0_25:0)	1.0742	0.3662	0.0001	654.6042	13.6009	↑	↓
Cer (d40:0)	2.7851	0.1794	0.0000	668.6198	14.1321	↑	↓
Cer (d15:0_25:0)	3.2254	0.0004	0.0000	668.6198	14.8120	↑	↓
Cer (d18:0_24:1)	1.8469	0.0499	0.0000	694.6355	14.0601	↑	↓
Cer (m39:0+O)	1.0743	0.3662	0.0001	654.6042	13.6500	↑	↓
CerP (m22:0_25:1)	1.3141	2.1368	0.0005	766.6837	15.8840	↓	↑
DG (12:0e_19:1)	2.9601	0.1365	0.0000	556.5299	10.2388	↑	↓
PE (16:0p_18:2)	1.3973	0.5033	0.0000	700.5276	9.9867	↑	↓
SM (d18:0_16:0)	1.5066	0.1065	0.0000	705.5905	9.7313	↑	↓
TG (16:1_16:1_18:2)	1.6214	1.5854	0.0059	844.7389	16.0130	↓	↑
TG (18:1_14:1_18:3)	1.1571	3.1182	0.0041	842.7232	15.5959	↓	↑
ZyE (33:5)	5.3497	7.8036	0.0165	868.7905	15.5900	↓	↑
ZyE (35:6)	27.5059	4.7335	0.0017	894.8062	15.6732	↓	↑
ZyE (37:6)	6.6748	2.8076	0.0134	922.8375	16.0493	↓	↑

Notes: (↑):Upregulated (↓): Downregulated. ^a Trends of the group M compared with the group C of the lipid metabolites. ^b Trends of the group T compared with the group M of the lipid metabolites.

Abbreviations: VIP, variable influence on projection; FC, fold change; P, p-value; Cal Mz, calculators mass to charge ratio; RT, retention time; Cer, ceramides; CL, cardiolipin; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; CerP, ceramides triglyceride; ZyE, zymosterol.

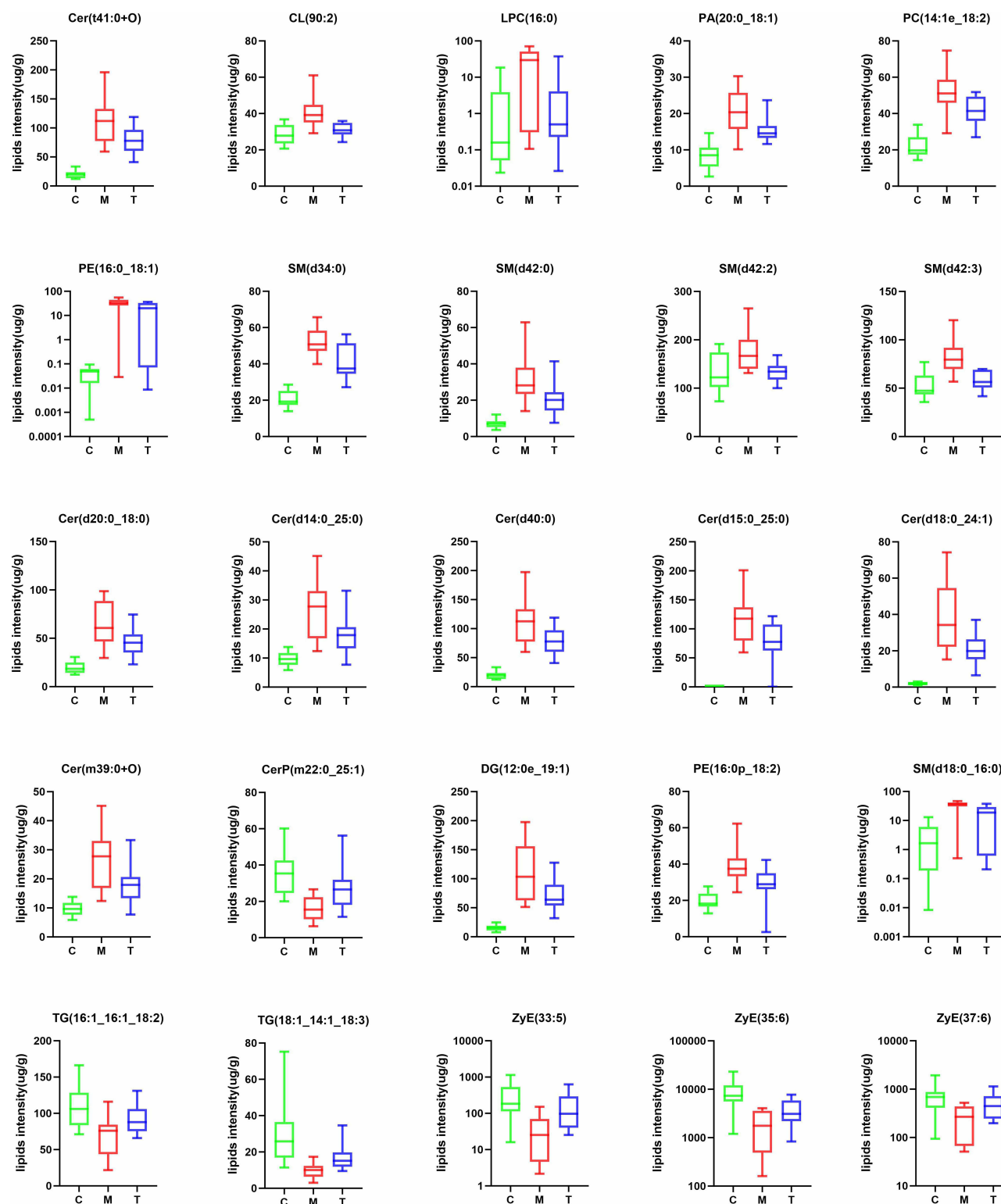


Figure 8 The box plot was employed to show changes of 25 lipid metabolites normalized by isotretinoin in the rabbit acne model treated.

regulating cell cycle processes. Previous evidence demonstrated that PC species can regulate the differentiation of keratinocytes.³⁴ Therefore, the alteration in PC levels suggests a defect in the process, resulting in disturbed keratinization.

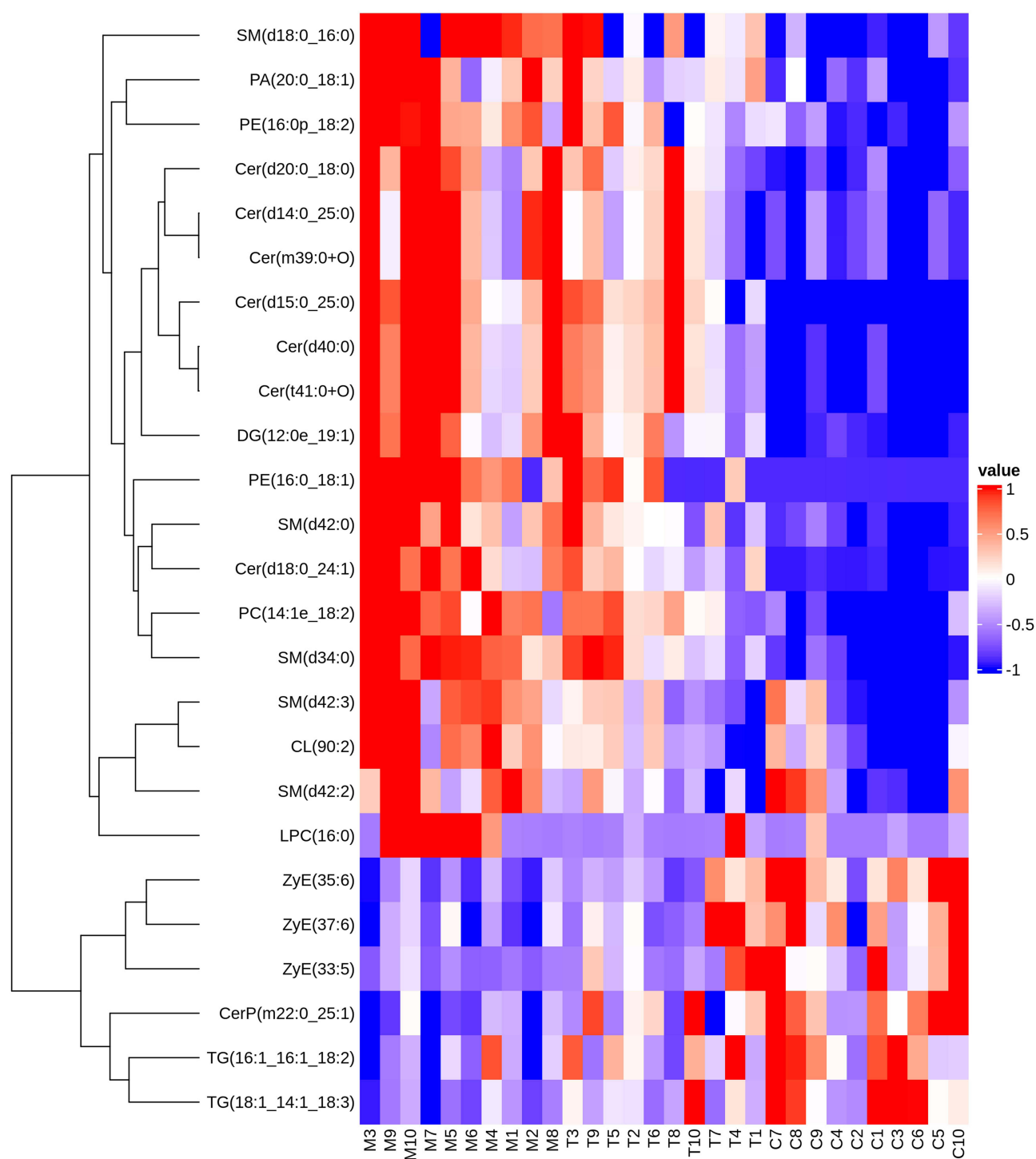


Figure 9 Hierarchical clustering heat map of 25 differential lipids between group C, group M and group T. Color scale on the right of the heat map represented normalized values of each identified lipid metabolites content. Red represents significant upregulation, blue represents significant downregulation.

SM is an abundant phospholipid in the cell membranes and lipoproteins of vertebrate animals. It plays a vital role as a key component of membrane rafts, influencing cell adhesion, cell signaling, membrane trafficking, and molecular sorting.^{35,36} Our data indicates that all 12 SM species were increased in group M, consistent with our previous untargeted metabolomics study that showed alteration of SM (d18:1/18:0).¹⁶ Additionally, Kaya et al found higher circulating levels of C16 SM in acne patients compared to healthy controls.³⁷ Previous research has demonstrated SM can stimulate the

proliferation of keratinocytes in vitro. Therefore, we hypothesize that the elevated levels of skin SMs may contribute to the development of acne by influencing epidermal keratinization processes.

Lipid Metabolites Normalized by Isotretinoin in the Rabbit Acne Model

In this study, 25 lipid metabolites were normalized by isotretinoin, suggesting a partial reversal of lipid metabolism dysregulation in the acne model. A previous untargeted metabolomics study identified three lipids molecules that were normalized by isotretinoin, including PC 18:1, PC (16:0/16:0), and LPC 18:1.¹⁶ Thus, this lipidomics study provides new insight into discovering potential lipid biomarkers for isotretinoin treatment. Notably, three ZyE species including ZyE (33:5), ZyE (35:6), and ZyE (37:6) were normalized by isotretinoin. Zymosterol is a precursor of cholesterol. Cholesterol is a key component of the cell membrane and constitutes up to 30% of total membrane lipids.³⁸ The synthesis of cholesterol in the skin is essential for maintaining the water permeability barrier. Acne vulgaris is associated with intrinsic abnormalities in epidermal barrier functions.³⁹ We speculate that isotretinoin may affect the skin barrier by regulating ZyE metabolism, potentially contributing to acne treatment.

This study had some limitations. The major limitation is the animal model used in this study is an ACNE-LIKE model. Hence, the model and findings may not translate to human acne. Another limitation is the relatively short observation period. Future studies involving a longer observation period are warranted.

Conclusions

Based on lipidomics, we demonstrated there were significant differences in skin lipid profiles between the acne model group and the control group. Besides, 25 lipid metabolites were normalized by isotretinoin. Our findings provide new insights into the role of lipid metabolism in the pathogenesis of acne and the action mechanism of isotretinoin.

Abbreviations

AV, acne vulgaris; UHPLC-Q-TOF MS, ultra-high-performance liquid chromatography and Q-TOF mass spectrometry; NH₄AC, ammonium acetate; NH₄F, ammonium fluoride; OPLS-DA, orthogonal partial least-squares discriminant analysis; VIP, variable importance in the projection; AUC, area under the curve; ROC, receiver operating characteristic; FC, fold Change; Cer, ceramides; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; ZyE, zymosterol; TG, triglyceride; DG, diglyceride; Hex1Cer, hexosyl ceramide; CerP, ceramides phosphate; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; CL, cardiolipin; FA, fatty acid.

Institutional Review Board Statement: All animal experiments were performed according to the regulation of institutional guidelines for the care and use of experimental animals and approved by the Animal Ethical Committee of The Second Affiliated Hospital of Nanchang University.

Data Sharing Statement

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Informed Consent Statement

Not applicable.

Acknowledgments

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest.

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