Zora Biosciences Oy

# Lipidomic characterization of ApoE(-/-) mouse tissue and plasma samples after smoke exposure

| | |
|---|---|
| **Sponsor** | Philip Morris International R&D |
| **Test site** | Zora Biosciences Oy |
| **Zora Study No** | **51811** |
| **Principal Investigator** | Mika Hilvo |
| **Bioanalytical Monitor** | Kim Ekroos |
| **Report Status** | Final Report |
| **Date** | October 23, 2014 |

© Zora Biosciences | www.zora.fi

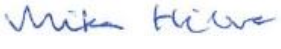
# 1 STUDY SYNOPSIS

| | |
|---|---|
| **STUDY TITLE** | Lipidomic characterization of ApoE(-/-) mouse tissue and plasma samples after smoke exposure |
| **TEST SITE** | Zora Biosciences Oy |
| **PURPOSE** | To study the effect of 3R4F and P1 tobacco products on the lipidome of mouse plasma, lung, liver, abdominal aorta and aortic arch tissue samples. |
| **STUDY DESIGN** | ApoE deficient mice were followed for 8 months. In three groups the exposure lasted for 8 months, i.e. the study had fresh air (Sham), 3R4F and P1 groups. Cessation and switch (from 3R4F to P1) was performed for additional two groups at 2 month time point. |
| **ANALYTICS** | Shotgun, ceramide, free cholesterol and triacylglycerol lipidomics were performed for selected samples and time points. |
| **RESULTS** | In lung, the results were consistent with previous studies showing high upregulation of phospholipids, especially phosphatidylglycerols in the 3R4F group. In plasma, triacylglycerols were elevated in the 3R4F group. Consistent with the previous studies specific ceramides in plasma were decreased in the 3R4F group. The results from liver were challenging to interpret, partly due to high variation in the sham group. In abdominal aorta the clearest results was the increase of cholesteryl esters due to 3R4F exposure. However, aortic arch showed upregulation of several lipid classes and therefore much clearer results than the abdominal aorta. |
| **CONCLUSIONS** | In lung, plasma and aortic arch 3R4F exposure caused lipidomic changes that were reversed by cessation or switch to P1 exposure, and the results were to large extent consistent with the previous studies. |

# 2 APPROVAL OF REPORT

| | |
|---|---|
| **Study number (Zora)** | 51811 |
| **Study Title (Zora)** | Lipidomic characterization of ApoE(-/-) mouse tissue and plasma samples after smoke exposure |
| **Test Site** | Zora Biosciences Oy |
| **Principal Investigator (Zora)** | Mika Hilvo, Senior Scientist, Ph.D. |
| **Bioanalytical Monitor (Zora)** | Kim Ekroos, Chief Technology Officer, Ph.D. |
| **Sponsor** | Philip Morris International R&D |
| **Project Leader (Sponsor)** | Julia Hoeng, PhD |

## SIGNATURES

| | | | |
|---|---|---|---|
| Signature: | | Date: | |
| Name: | | Company: | |
| Signature: |  | Date: | October 23, 2014 |
| Name: | Mika Hilvo, Senior Scientist, PhD. Principal Investigator | Company: | Zora Biosciences Oy |
| Signature: |  | Date: | October 23, 2014 |
| Name: | Kim Ekroos, Chief Technology Officer, PhD. Bioanalytical Monitor | Company: | Zora Biosciences Oy |

# 3 TABLE OF CONTENTS

1 STUDY SYNOPSIS ..... 2
2 APPROVAL OF REPORT ..... 3
3 TABLE OF CONTENTS ..... 4
4 INFORMATION ON THE SPONSOR ..... 7
5 INFORMATION ON THE TEST SITE ..... 7
6 INTRODUCTION ..... 8
7 AIMS AND DELIVERABLES ..... 8
8 SPECIFICS OF THE STUDY ..... 9
8.1 Study title ..... 9
8.2 Study design ..... 9
8.3 Study type ..... 10
8.4 Biological samples ..... 10
8.4.1 Sample shipment ..... 10
8.4.2 Sample handling, storage and destruction ..... 10
8.5 Test items ..... 10
8.6 Type and number of reference items ..... 10
8.7 Stability of test and reference items ..... 10
8.8 Reagents ..... 10
9 SPECIFICS OF THE METHODS ..... 11
9.1 Sample preparation and homogenization ..... 11
9.2 Lipid extraction ..... 11
9.3 Mass spectrometric analyses ..... 12
9.4 Data processing ..... 12
9.4.1 The MS data ..... 12
9.5 Acceptance criteria for the results ..... 13
9.5.1 Quality controls ..... 13

9.5.2 Quantification range ..... 13
9.5.3 Analysis acceptance criteria ..... 13
9.6 Statistical analysis ..... 13
10 RESULTS ..... 14
10.1 General notes on results presentation ..... 14
10.2 Comparisons at 2 month time point ..... 14
10.2.1 Lung ..... 14
10.2.2 Liver ..... 16
10.2.3 Abdominal aorta ..... 18
10.2.4 Summary ..... 18
10.3 Comparisons at 3 month time point ..... 19
10.3.1 Lung ..... 19
10.3.2 Liver ..... 22
10.3.3 Abdominal aorta ..... 26
10.3.4 Summary ..... 29
10.4 Comparisons at 8 month time point ..... 30
10.4.1 Plasma ..... 30
10.4.2 Lung ..... 32
10.4.3 Liver ..... 36
10.4.4 Abdominal aorta ..... 40
10.4.5 Aortic arch ..... 42
10.4.6 Summary ..... 45
10.5 Pairwise group comparisons: cessation against 3R4F at 2 months ..... 45
10.5.1 Lung ..... 45
10.5.2 Liver ..... 48
10.5.3 Abdominal aorta ..... 51
10.5.4 Summary ..... 53
10.6 Pairwise group comparisons: switch against 3R4F at 2 months ..... 53
10.6.1 Lung ..... 53

10.6.2 Liver ..... 57
10.6.3 Abdominal aorta ..... 60
10.6.4 Summary ..... 61
10.7 Pairwise time point comparisons: 3 and 8 vs. 2 months ..... 61
10.7.1 Lung ..... 61
10.7.2 Liver ..... 65
10.7.3 Abdominal aorta ..... 68
10.7.4 Summary ..... 70
10.8 Pairwise time point comparisons: 8 vs. 3 months ..... 70
10.8.1 Lung ..... 70
10.8.2 Liver ..... 73
10.8.3 Abdominal aorta ..... 75
10.8.4 Summary ..... 77
11 SUMMARY AND DISCUSSION ..... 77
11.1 Plasma ..... 77
11.2 Lung ..... 78
11.3 Liver ..... 79
11.4 Abdominal aorta ..... 80
11.5 Aortic arch ..... 80
12 ARCHIVING ..... 81
13 REFERENCES ..... 81
14 APPENDICES ..... 82

# 4 INFORMATION ON THE SPONSOR

| | |
|---|---|
| **Sponsor** | Philip Morris International R&D<br>Systems Toxicology<br>Biological Systems Research |
| **Project Leader** | Julia Hoeng, PhD |

# 5 INFORMATION ON THE TEST SITE

| | |
|---|---|
| **Test site** | Zora Biosciences Oy<br>Biologinkuja 1<br>FI-02150 Espoo<br>Finland |
| **Principal Investigator** | Mika Hilvo, PhD<br>Tel: +358 50 534 7782<br>E-mail: mika.hilvo@zora.fi |
| **Bioanalytical monitor** | Kim Ekroos, PhD<br>Tel: +358 40 744 8997<br>E-mail: kim.ekroos@zora.fi |

# 6 INTRODUCTION

Abdominal aorta (n=104), liver left lobe (n=104), lung right lobe (n=104), aortic arch (n=50) and plasma (n=40) samples obtained from ApoE knockout mice exposed to cigarette smoke were provided from Philip Morris International R&D to Zora Biosciences Oy. Lipidomics analyses of these samples were conducted by Zora Biosciences Oy.

# 7 AIMS AND DELIVERABLES

The deliverables of the study were to analyze the lipid profiles of the samples.

Lipidomics analysis of the study samples included total concentrations of lipid classes and molecular lipids when detected. The lipid classes monitored on selected lipidomics platforms are presented in **Table 1**.

**Table 1.** Lipid classes monitored per platform and tissue type.

| Platform | Monitored lipid classes | Tissue types analyzed |
|---|---|---|
| Shotgun Lipidomics (SG) | Cholesteryl esters (CE)<br>Phosphatidylcholines (PC)<br>Lysophosphatidylcholines (LPC) and other lysophospholipids (LPL)<br>Ether-linked phosphatidylcholines (PC O) and other ether-linked phospholipids (PL O)<br>Phosphatidylserines (PS)<br>Phosphatidylethanolamines (PE)<br>Phosphatidylglycerols (PG)<br>Phosphatidylinositiols (PI)<br>Phosphatidic acid (PA)<br>Sphingomyelins (SM)<br>Diacylglycerols (DAG) | Abdominal aorta, liver, lung, aortic arch |
| Free cholesterol quantification | Free cholesterol (FC) | Abdominal aorta (8 m time point), aortic arch, plasma |
| Triacylglycerol Lipidomics (TAG) | Triacylglycerols (TAG) | Liver, plasma |
| Ceramide and cerebroside Lipidomics (CER) | Ceramides (Cer)<br>Cerebrosides (Lactosylceramides, LacCer; Galactosyl- and Glucosylceramides, Gal/GlcCer, globotriaosylceramides, Gb3Cer) | Abdominal aorta, liver, lung, aortic arch, plasma |

# 8 SPECIFICS OF THE STUDY

## 8.1 Study title

Lipidomic characterization of ApoE(-/-) mouse tissue and plasma samples after smoke exposure

## 8.2 Study design

The aim of the study was to compare the effect of different smoke products on the lipidomic profiles of mouse tissue and plasma samples. Lipidomic analyses were performed on plasma, liver, lung, aortic arch and abdominal aorta samples obtained from ApoE knockout mice exposed to smoke for 2, 3 or 8 months, as described in **Table 2** and illustrated in **Figure 1**.

The groups were compared to each other within each tissue type and time point.

**Table 2.** Study design.

| Group number | Group name | Tissue | Number of samples |
|---|---|---|---|
| 1 | Sham | Plasma / liver / lung / abdominal aorta / aortic arch | 40 / 104 / 104 / 104 / 50 |
| 2 | 3R4F | Plasma / liver / lung / abdominal aorta / aortic arch | 40 / 104 / 104 / 104 / 50 |
| 3 | P1 | Plasma / liver / lung / abdominal aorta / aortic arch | 40 / 104 / 104 / 104 / 50 |
| 4 | Cessation | Plasma / liver / lung / abdominal aorta / aortic arch | 40 / 104 / 104 / 104 / 50 |
| 5 | Switch | Plasma / liver / lung / abdominal aorta / aortic arch | 40 / 104 / 104 / 104 / 50 |

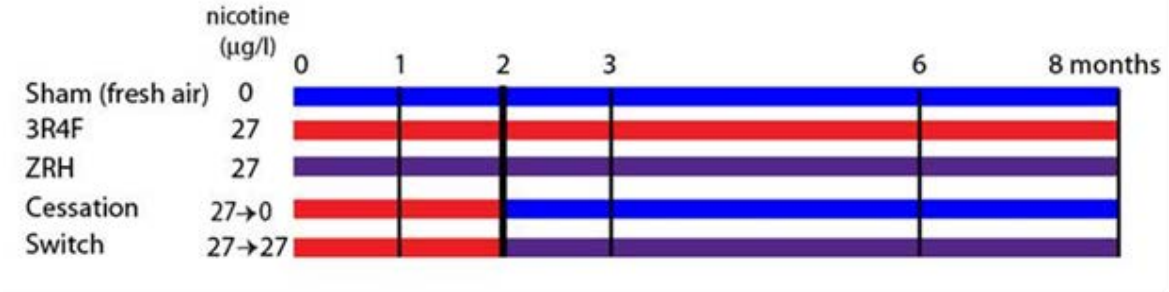



**Figure 1.** Illustration of the study design. The ZRH in the figure is referred to as P1 in this report.

## 8.3 Study type

This study was conducted according to the standard operating procedures of Zora Biosciences Oy.

## 8.4 Biological samples

### 8.4.1 Sample shipment

A confirmation e-mail to sender of samples was sent where it was confirmed which samples had arrived to Zora. This e-mail was printed, signed and put into a Study Package. In addition, a Zora Sample book was created where all the relevant information about the samples was collected. This Sample book was saved into a Study Package.

### 8.4.2 Sample handling, storage and destruction

Frozen samples were thawed on chilly environment upon starting the analysis in Zora. The samples were stored at -80°C prior to analysis with the correct project name and code label. All samples and lipid extracts will be destroyed after a year or as agreed with the customer.

## 8.5 Test items

Molecular lipid species of different lipid classes in mouse plasma and tissue samples.

## 8.6 Type and number of reference items

Lipid specific synthetic internal standards were prepared and used for lipid quantification. The stocks and working solutions of the reference standards were stored at -20°C.

## 8.7 Stability of test and reference items

All samples were stored at -80°C. The lipid extracts dissolved in appropriate solvents were stored at -20°C prior to analysis.

## 8.8 Reagents

The reagents used in this study were documented and saved in the specific Solvent Logbook of Zora Biosciences Oy. The information is available upon request.

# 9 SPECIFICS OF THE METHODS

The lipid analyses of plasma/tissue samples were performed according to the Standard Operating Procedures (SOP), the Lab Method Sheets (LMS), and data Processing Method Sheets (PMS) of Zora Biosciences Oy [1]. *The description of methods in this report is not optimal for the materials and methods section in scientific publications. When preparing possible scientific articles, please contact the principal investigator at Zora, who will provide a customized description for the materials and methods section.*

The used instruments, software, and methods are summarized in **Table 3**.

**Table 3.** Summary of used instruments, software, and detection methods.

| Method | Sample volume (µl) | Instrument | Software | Detection method |
|---|---|---|---|---|
| Lipid extraction | | Microlab Star Robot (Hamilton Robotics) | Microlab Vector V4.1.1 | |
| Shotgun and Free Cholesterol Lipidomics | 10 | TriVersa NanoMate (Advion Biosciences) | ChipSoft V8.1.0 | Precursor Ion Scan (PIS) |
| Triacylglycerol Lipidomics | 10 | QTRAP 5500 MS (ABSciex) | Analyst V1.6.2 LipidView V1.2 | |
| Ceramide Lipidomics | 10 | Eksigent 100-XL UHPLC system (Eksigent) QTRAP 5500/6500 MS (Applied Biosystems) | Analyst V1.6.2 MultiQuant V3.0 | Multiple reaction monitoring (MRM) |

## 9.1 Sample preparation and homogenization

Lung and liver samples were pulverized with CP02 CryoPrep Dry Pulverization System (Covaris), and resuspended in ice-cold methanol containing 0.1% butyl-hydroxy-toluene (BHT) in a concentration of 100 mg/ml. Due to the small amounts of the abdominal aorta and aortic arch, these tissues were extracted over-night following the procedure in Section 9.2, except the robotic part. The samples were mixed continuously using zirconium beads.

## 9.2 Lipid extraction

Lipids (ceramides, cholesterol, glycerolipids, and glycerophospholipids) were extracted using a modified Folch lipid extraction [1] performed on a Hamilton Microlab Star robot. Samples were spiked with known amounts of non-endogeneous synthetic internal standards. After lipid extraction, samples were reconstituted

in chloroform:methanol (1:2, v/v) and synthetic external standards were post-extract spiked to the extracts. The extracts were stored at -20°C prior to MS analysis.

For quantification of free cholesterol, an aliquot of each lipid extract was treated with acetyl chloride to derivatize FC to modified cholesteryl ester species.

## 9.3 Mass spectrometric analyses

In Shotgun Lipidomics, lipid extracts were analyzed using a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500) equipped with a robotic nanoflow ion source (NanoMate TriVersa) according to Ståhlman and colleagues [2]. Molecular lipids were analyzed in both positive and negative modes using Multiple Precursor Ion Scanning (MPIS) based methods [3], [4]. Triacylglycerols (TAG) were analyzed using Precursor Ion Scanning (PIS) and Neutral Loss scanning (NL) based methods [5]. The molecular lipid species were identified and quantified in semi-absolute or absolute amounts [2] according to the corresponding LMSs.

Targeted molecular lipids were analyzed on a hybrid triple quadrupole/linear ion trap mass spectrometer (5500 QTRAP) equipped with an ultra-high pressure liquid chromatography (UHPLC) system (Eksigent 100-XL) using multiple reaction monitoring (MRM) –based method in negative ion mode according to the corresponding LMS.

## 9.4 Data processing

All software used in the data analysis is summarized in **Table 4**.

**Table 4.** Summary of the software used in the data analysis

| Software | Version | Instrument / Data analysis |
|---|---|---|
| LipidView™ | 1.2 | Processing of QTRAP data |
| MultiQuant™ | 3.0 | Processing of QTRAP data |
| Analyst | 1.6.2 | Analytical software |
| SAS | 9.3 | Data analysis |
| Tableau Desktop | 8.2 | Data analysis |

### 9.4.1 The MS data

The mass spectrometry data obtained from MS instruments were exported as .wiff or .txt files that represent the basic raw files of lipidomic analysis. These files contain information on masses of identified molecules and their counts (intensities and areas). Masses and counts of detected peaks were converted into a list of corresponding lipid names and concentrations.

Calibration lines were generated to determine the dynamic quantification range for each lipid monitored, *e.g.*, the quantification limits. As the internal standards used behave in the same way as endogenous lipids they were used for quantifying endogenous lipids.

The MS data files were processed using LipidView™ 1.2 or MultiQuant™ 2.0 for producing a list of lipid names and peak areas. Lipids were normalized to their respective internal standard (the peak area of the endogenous lipid was divided by the peak area of the corresponding internal standard) and sample volume. The concentrations of molecular lipids are presented as pmol/µl or pmol/µg tissue wet weight for plasma or tissue samples, respectively.

## 9.5 Acceptance criteria for the results

### 9.5.1 Quality controls

The ratio of synthetic Internal Standards (IS) to corresponding post-extract spiked External Standards (ES), and MS analysis of extracted matrix and solvents served as quality controls (QC) of the analysis. In addition, extracted reference plasma/tissue samples were analyzed for monitoring the instruments' performance.

### 9.5.2 Quantification range

Calibration lines using synthetic standards were obtained prior to sample analysis. The calibration lines consist of a minimum of four accepted standard points covering the linear quantification range. The calibration lines were used to determine the quantification limits of the method.

### 9.5.3 Analysis acceptance criteria

The analysis was accepted based on the linearity of the calibration lines. The linear regression must exceed 0.95 based on at least four out of six non-zero standards.

The analysis is accepted based on the identification of sample specific IS.

## 9.6 Statistical analysis

Unpaired Student t-test was performed on log-transformed concentrations to calculate p-values. Group differences are reported as relative percentage differences of average concentrations of the groups.

The results of statistical analyses are shown as heatmap presentations for the study groups. In some figures it seems that there are missing values, however, that may indicate that the changes between groups were negligible or in comparison group(s) the lipids were beyond the quantification limit.

Figures show significance of values as *p≤0.05, ** p≤0.01 or ***p≤0.001. Red and blue colors indicate that the level of lipid is increased or decreased, respectively, in the comparison group. The relative difference is encoded by color intensity. For example, if a change in lipid concentration in Group 2 versus Group 1 is encoded in red, it means that concentration of this lipid is higher in Group 2 than in Group 1.

# 10 RESULTS

## 10.1 General notes on results presentation

This Report provides lipidomic data on all molecular lipids analyzed with a single technical replicate in each sample. All lipids detected by mass spectrometry analyses in the study samples were identified and quantified as described in the Methods section of this report. The Full Data Table of all detected lipid species and their concentrations in each study sample are provided in Appendix 1. The results of the statistical analyses are provided in Appendix 2-4. For data visualization, results are additionally provided as a Tableau file (Appendix 5).

The shotgun data from liver samples showed a small variation between the runs, which affected mainly phospholipids. Due to this, the shotgun results for liver were corrected by using the median values of the quality control samples in the plates.

## 10.2 Comparisons at 2 month time point

### 10.2.1 Lung

**Platforms: SG, CER**

**Figure 2** summarizes the effect of 3R4F and P1 cigarette smokes as the relative changes (as described in the materials and methods section 9.6), presented as heatmaps where blue and red bars denote decreased or increased concentration, respectively, of the most significantly changed lipids (due to large number of changing lipids only p<0.001 are shown) in the 3R4F and P1 groups at 2 month time point. As shown in the figure and evident from the Tableau appendix, 3R4F group had increased levels of ceramides, diacylglycerols, glucosyl/galactosylceramides and lactosylceramides (cerebrosides), phosphatidylcholines and phosphatidylglycerols, and in general these lipid classes were not changed in the P1 group. Total PA showed high increase in both groups, but this was mainly due to PA 18:0/22:4, which was absent in the Sham group. Interestingly, in the lung the PGs were highly increased in the 3R4F group, while only a few PGs showed statistically significant increase (p<0.05) in the P1 group (not shown in Figure 1). The major

changes observed in the P1 group were the increase of cholesteryl esters (CEs) and the decrease of phosphatidylethanolamines (PEs).

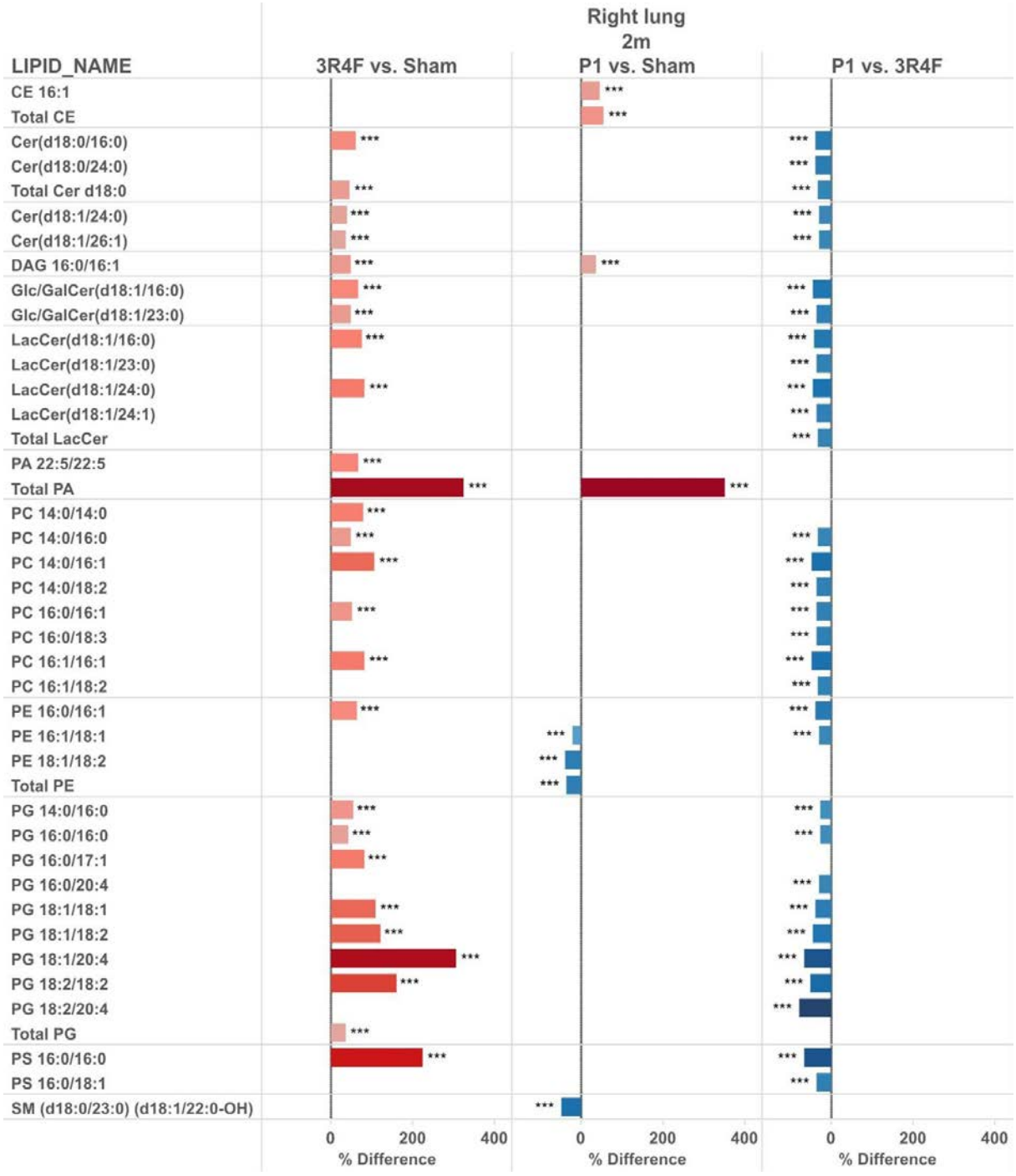



**Figure 2.** Relative percentage differences in lung at 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.2.2 Liver

**Platforms: SG, TAG, CER**

The relative differences of sphingolipids for both (3R4F and P1) groups are shown in **Figure 3**. In both groups there was a decrease in globotriasoylceramides (Gb3) and cerebrosides when compared to the Sham group. The results for other lipids are shown in **Figure 4**. Especially in the 3R4F group triacylglycerols were decreased and phosphatidylcholines increased. In addition, both 3R4F and P1 groups showed increase in phosphatidylglycerols.

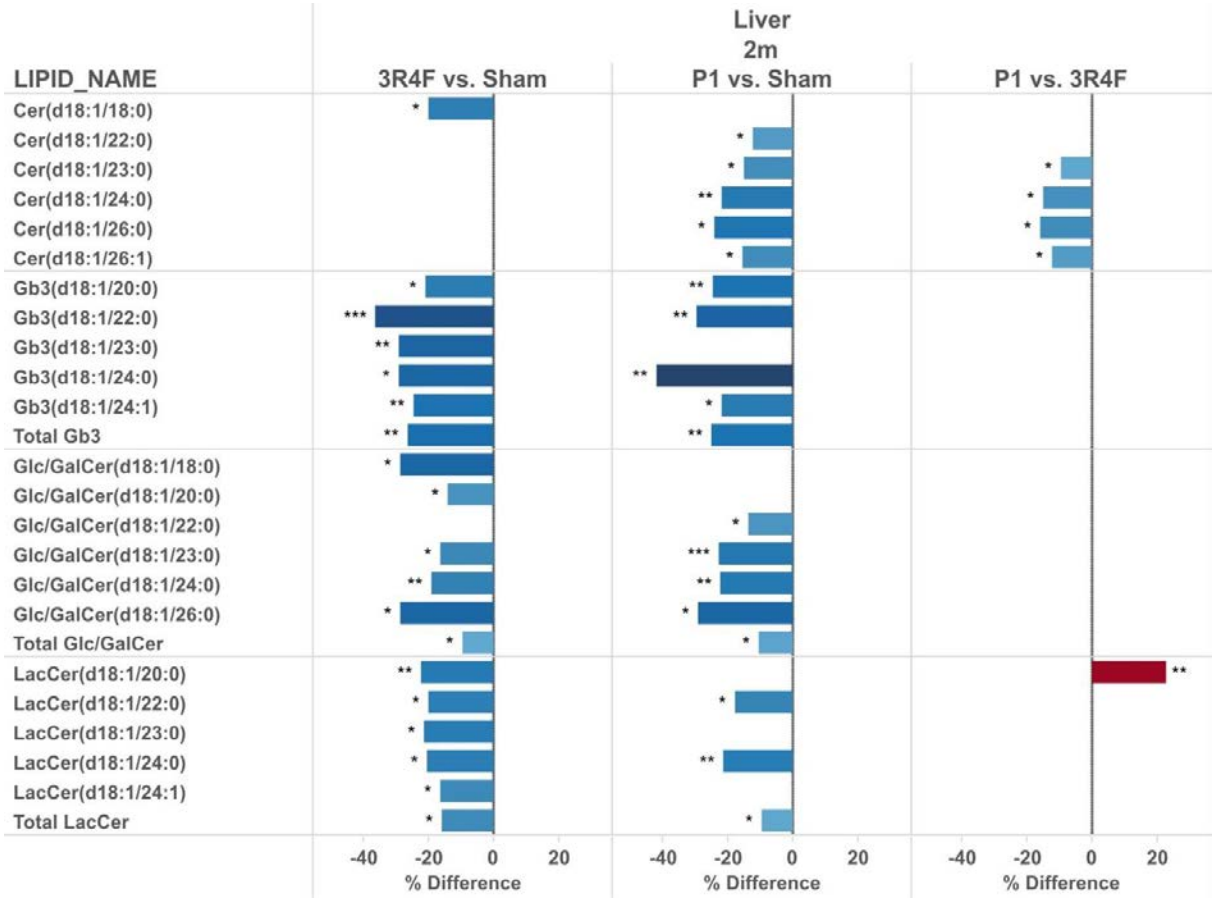



**Figure 3.** Relative percentage differences in liver at 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

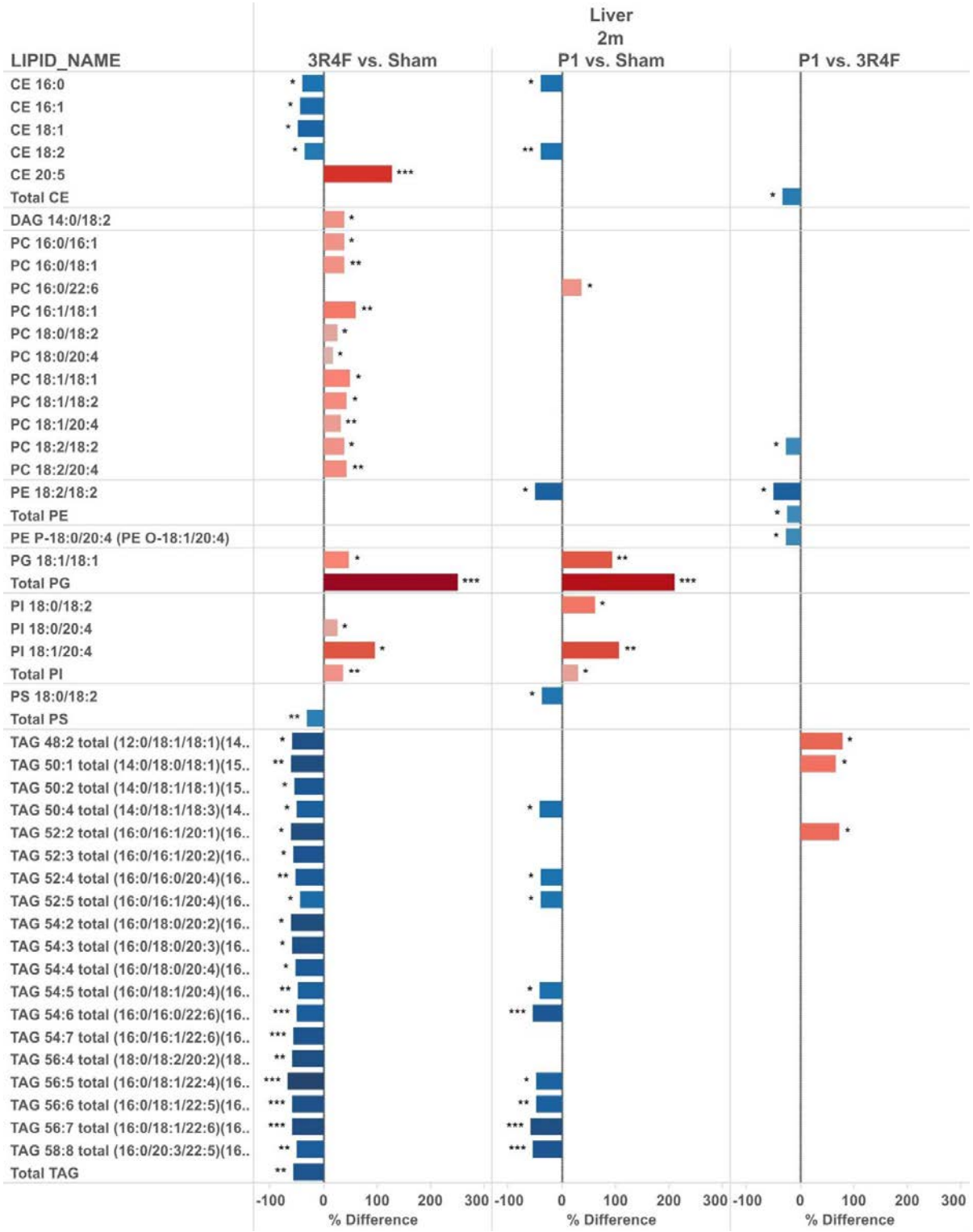



**Figure 4.** Relative percentage differences in liver at 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.2.3 Abdominal aorta

**Platforms: SG, CER**

In the abdominal aorta there were no statistically significant differences for ceramides at 2 month time point. The shotgun results are presented in **Figure 5**. In the shotgun data the P1 group showed the largest changes, as e.g. the sphingomyelins and phosphatidylserines were decreased.

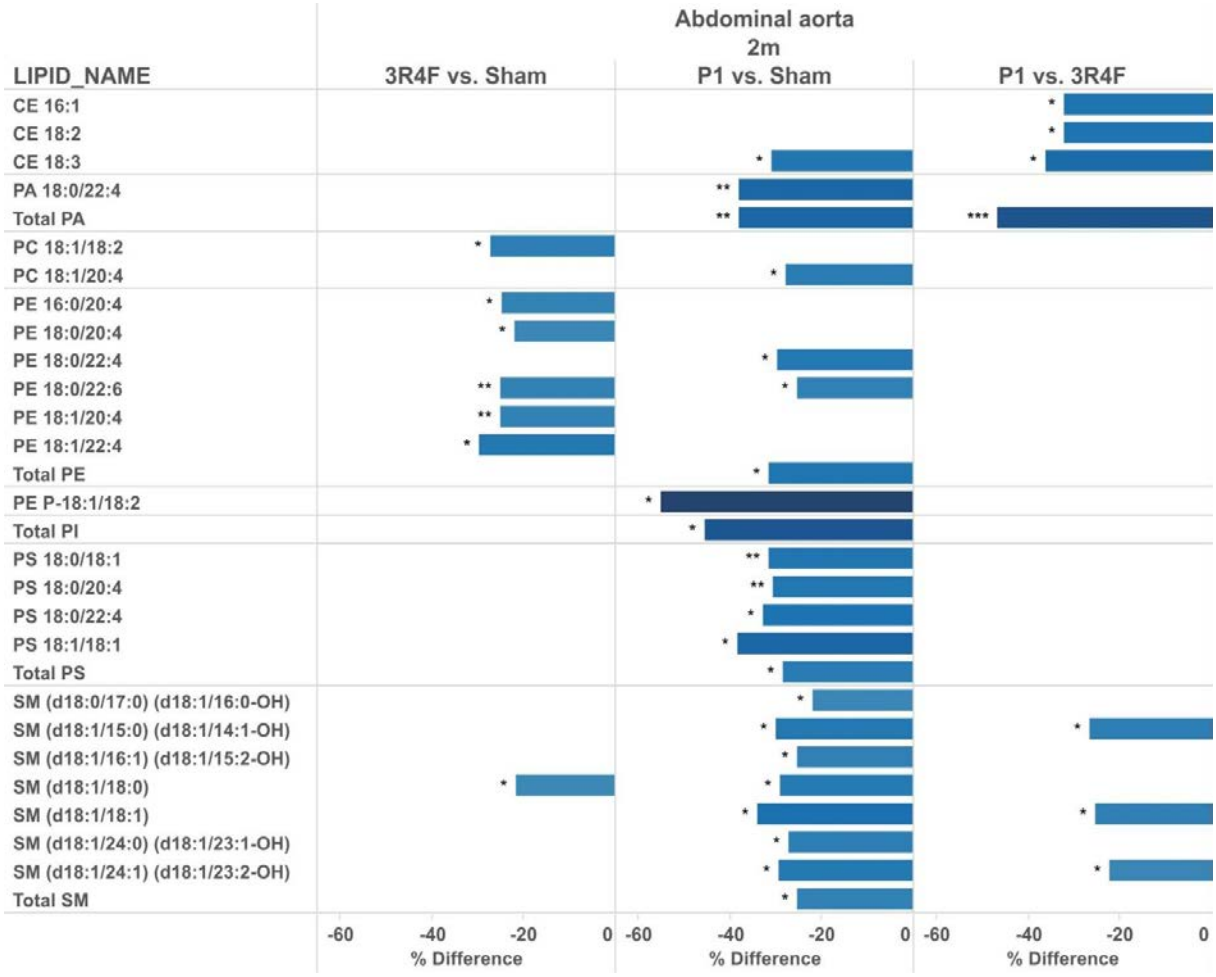



**Figure 5.** Relative percentage differences in liver at 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.2.4 Summary

At 2 month time point there was an increase in several lipid classes in the lung of the 3R4F group, which was not observed in the P1 group. In liver, there was a decrease of sphingolipids in both groups, and decrease of triacylglycerols and increase of diacylglyerols especially in the 3R4F group. In abdominal aorta P1 group showed the greatest changes with decreasing sphingomyelins and phosphatidylserines.

## 10.3 Comparisons at 3 month time point

### 10.3.1 Lung

**Platforms: SG, CER**

The relative differences for all the groups as compared to the Sham group are shown in **Figure 7** (lipids with p<0.001 are shown). In contrast to the 2 month results, the increase of ceramides was not observed in the 3R4F group, and instead decrease of ceramides and lactosylceramides was evident in the P1 group. Consistent with the 2 month time point findings, phosphatidylcholines, and especially phosphatidylglycerols were increased in the 3R4F group. However, these changes decreased both in the cessation and switch groups, and also the level of the most significantly altered PG, PG 18:1/20:4, showed 400 % increase in the 3R4F group but lower 200% increase in the cessation and switch groups. The levels of all PGs are shown in **Figure 6**.

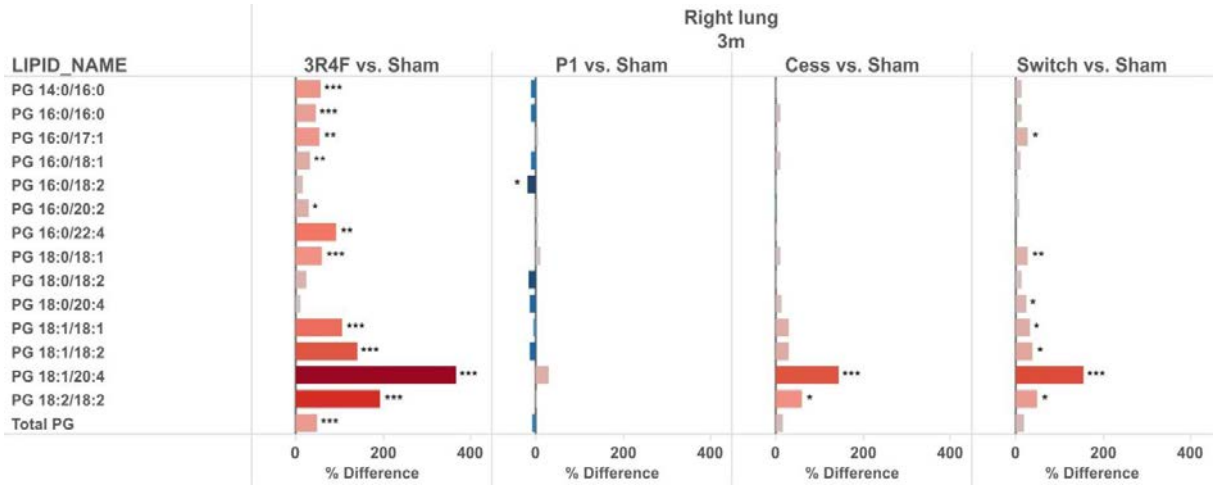



**Figure 6.** Relative percentage difference of phosphatidylglycerols in lung at 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

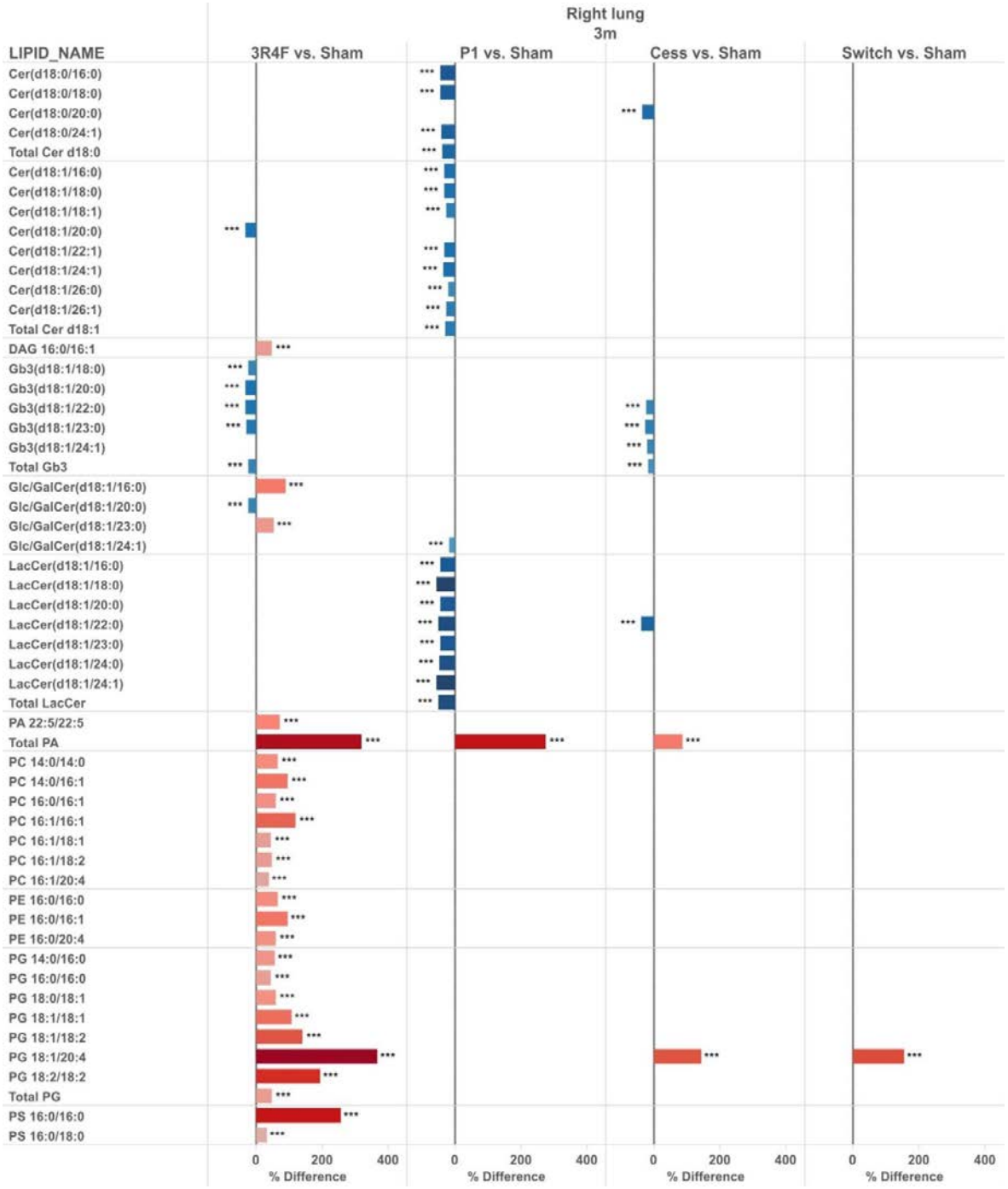



**Figure 7.** Relative percentage differences in lung at 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

When comparing the P1, cessation and switch groups to the 3R4F group at 3 month time point, interestingly the most dramatic changes were observed for the P1 group (**Figure 8**, only p<0.001 are shown). However, most of the changes were consistent in all groups, as phospholipids, i.e. phosphatidylcholines, phosphatidylethanolamines and especially phosphatidyglycerols were decreased in the other groups as

compared to the 3R4F group. In addition, ceramides were decreased especially in the P1 group and cholesterylesters were increased only in that group.

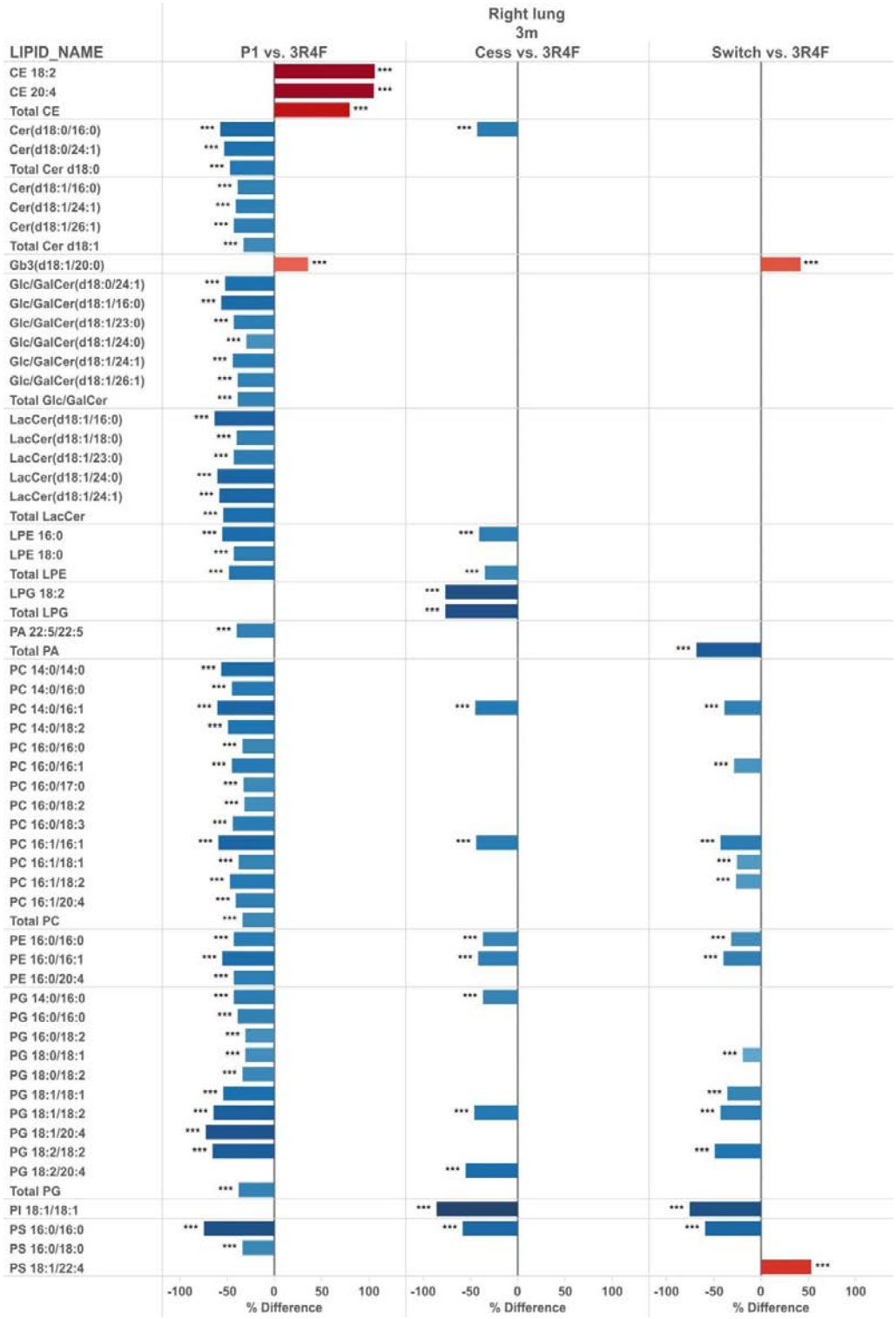



**Figure 8.** Relative percentage differences in lung at 3 month time point when compared to the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.3.2 Liver

**Platforms: SG, TAG, CER**

The relative difference of ceramides for all the groups as compared to the Sham group are shown in **Figure 9** and for other lipids in **Figure 10**. Consistent with the 2 month results, globotriaosylceramides and cerebrosides were decreased in the 3R4F group. However, these changes were not so strong in the P1 group, although there was an overall decreasing trend in that group as well. The results in the cessation and switch groups resembled more those of the 3R4F group than the P1 group. In addition, in the cessation and switch groups d18:1-ceramides showed statistically significant decrease.

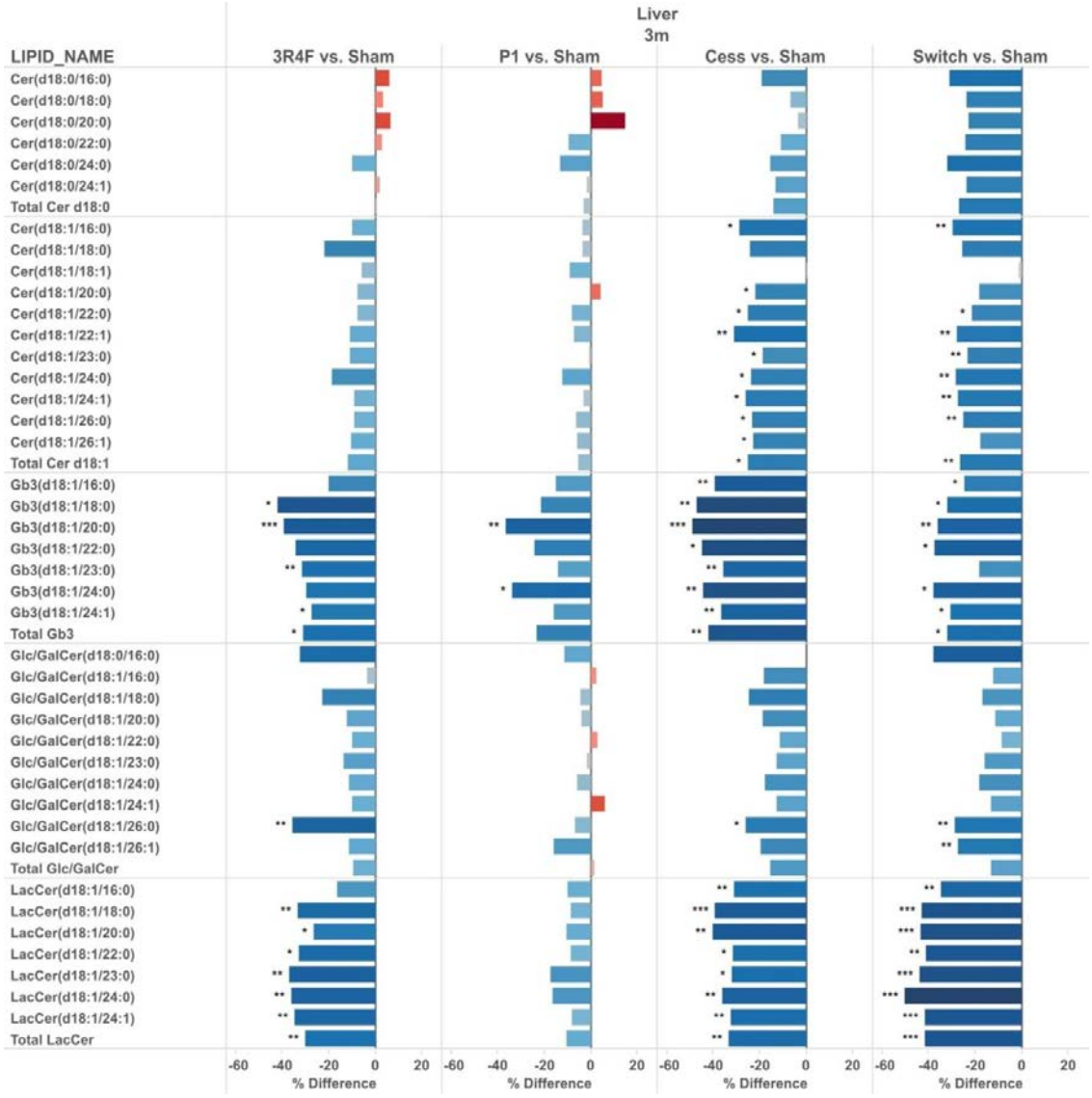



**Figure 9.** Relative percentage differences of ceramides in liver at 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

Regarding the other lipidomic changes, 3R4F group showed increased phosphatidic acid, and in the cessation and switch groups diacylglycerols were increased. Triacylglycerols were decreased in all groups.

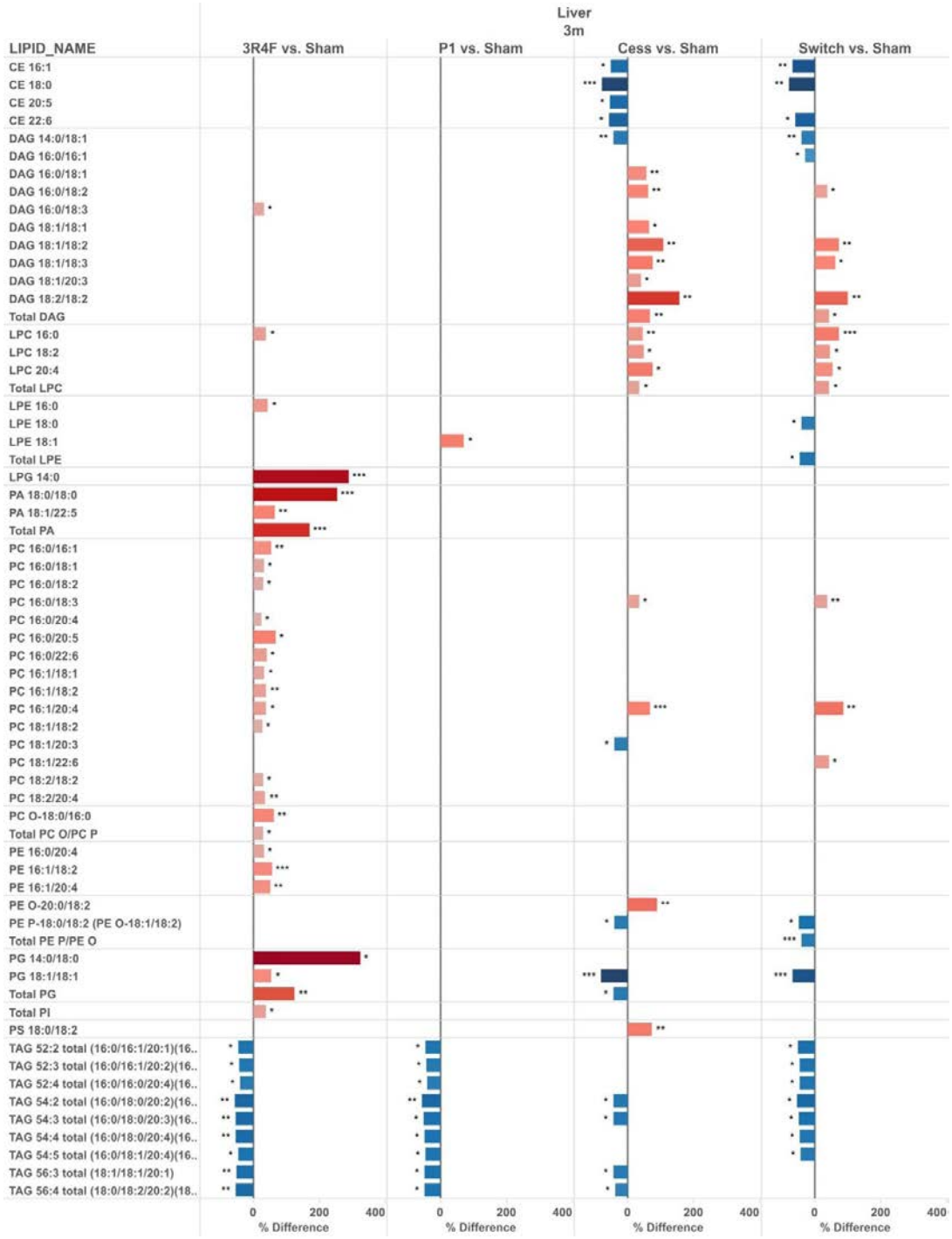



**Figure 10.** Relative percentage differences of ceramides in liver at 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

As shown in **Figure 11**, there were rather modest changes in ceramides when the comparison against the 3R4F group was performed. The P1 group showed increase of lactosylceramides and the switch group decrease of few ceramides and lactosylceramides.

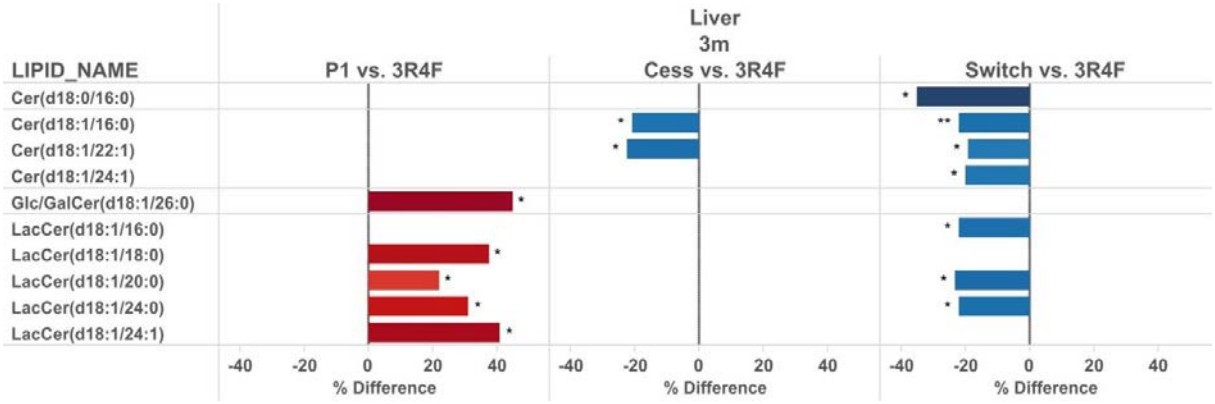



**Figure 11.** Relative percentage differences in liver at 3 month time point when compared to the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

In contrast to the ceramides, the other lipids showed more dramatic changes when the comparison against the 3R4F group was performed **(Figure 12)**. In the P1 group there were only a few changes whereas in the cessation and switch group there was a decrease in phospholipids and increase in diacylglyerols containing at least one 18:1 fatty acyl side chain.

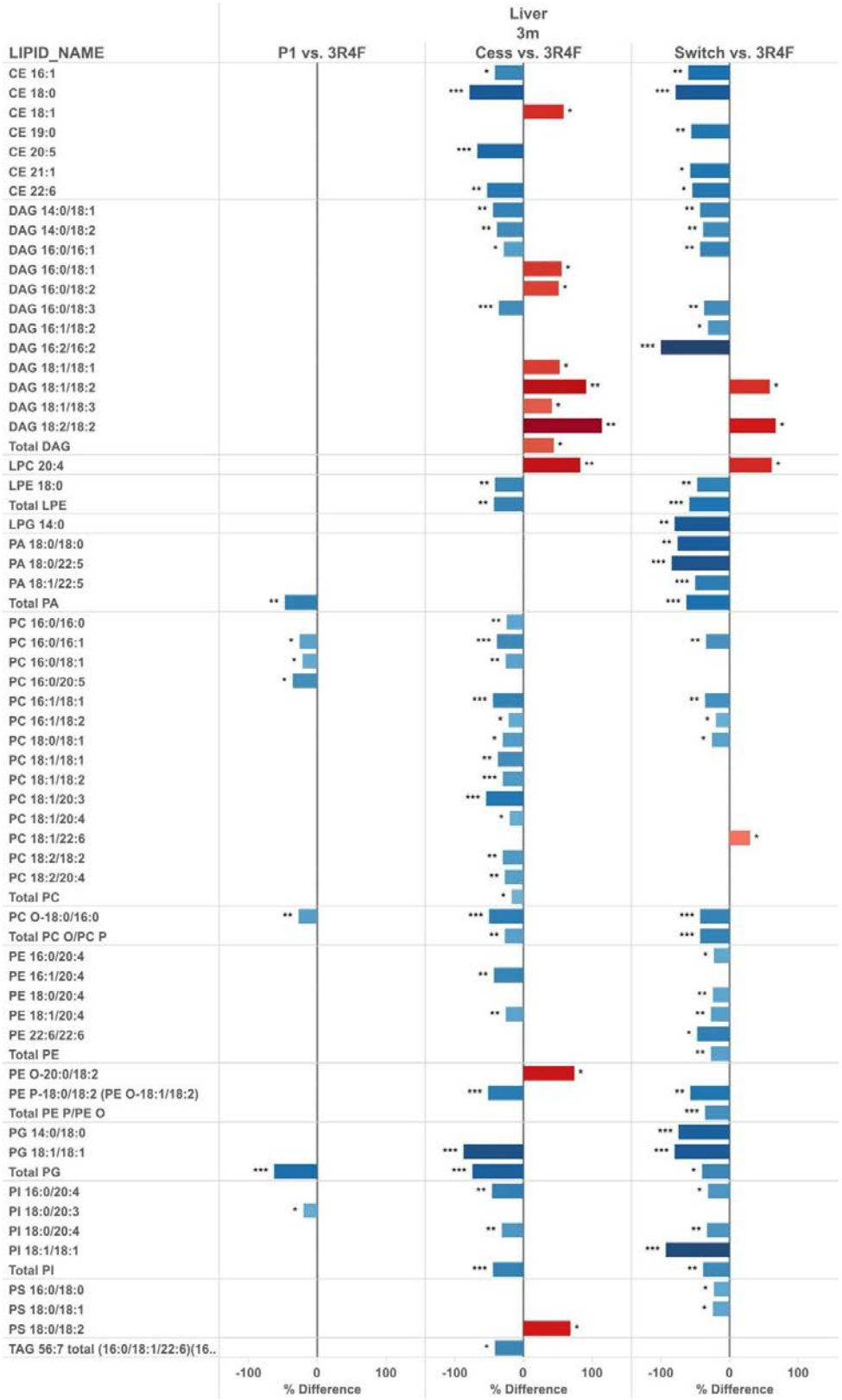



**Figure 12.** Relative percentage differences in liver at 3 month time point when compared to the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.3.3 Abdominal aorta

**Platforms: SG, CER**

The relative difference of ceramides for all the groups as compared to the Sham group are shown for ceramides in **Figure 13** and for other lipids in **Figure 20**. For ceramides, in the 3R4F and P1 groups there were only occasional statistical significant differences, whereas in the switch and especially in the cessation group ceramides and glycoceramides were increased. Regarding other lipids, phosphatidylcholines and phosphatidylethanolamines were decreased in the 3R4F group.

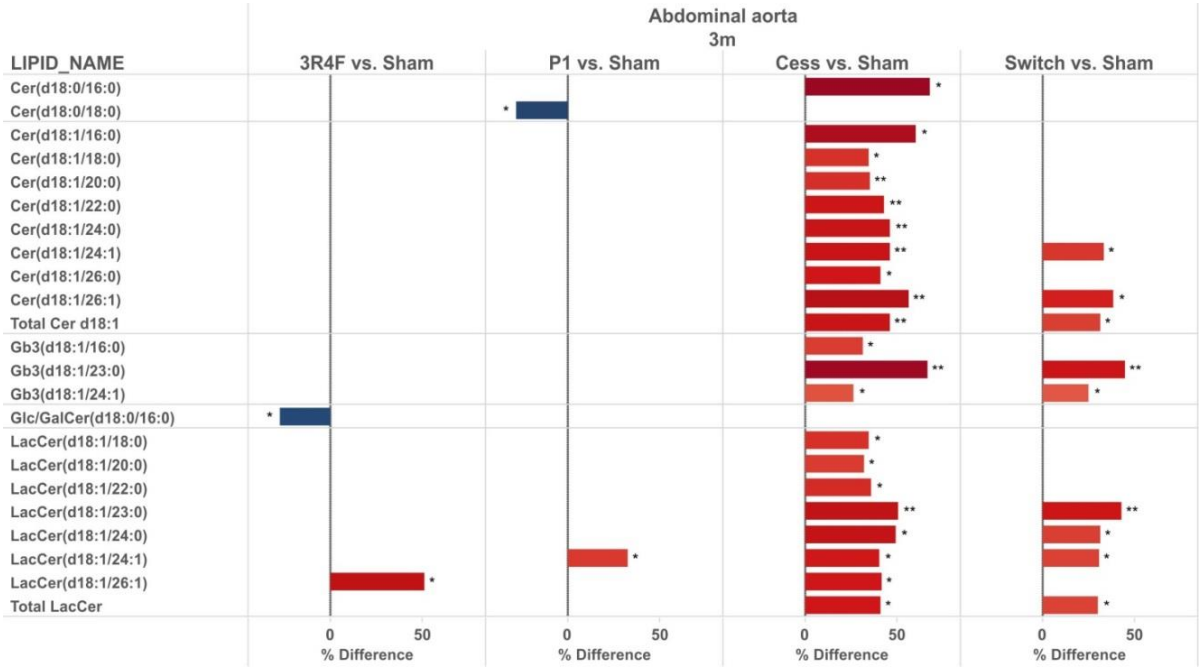



**Figure 13.** Relative percentage differences of ceramides in abdominal aorta at 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

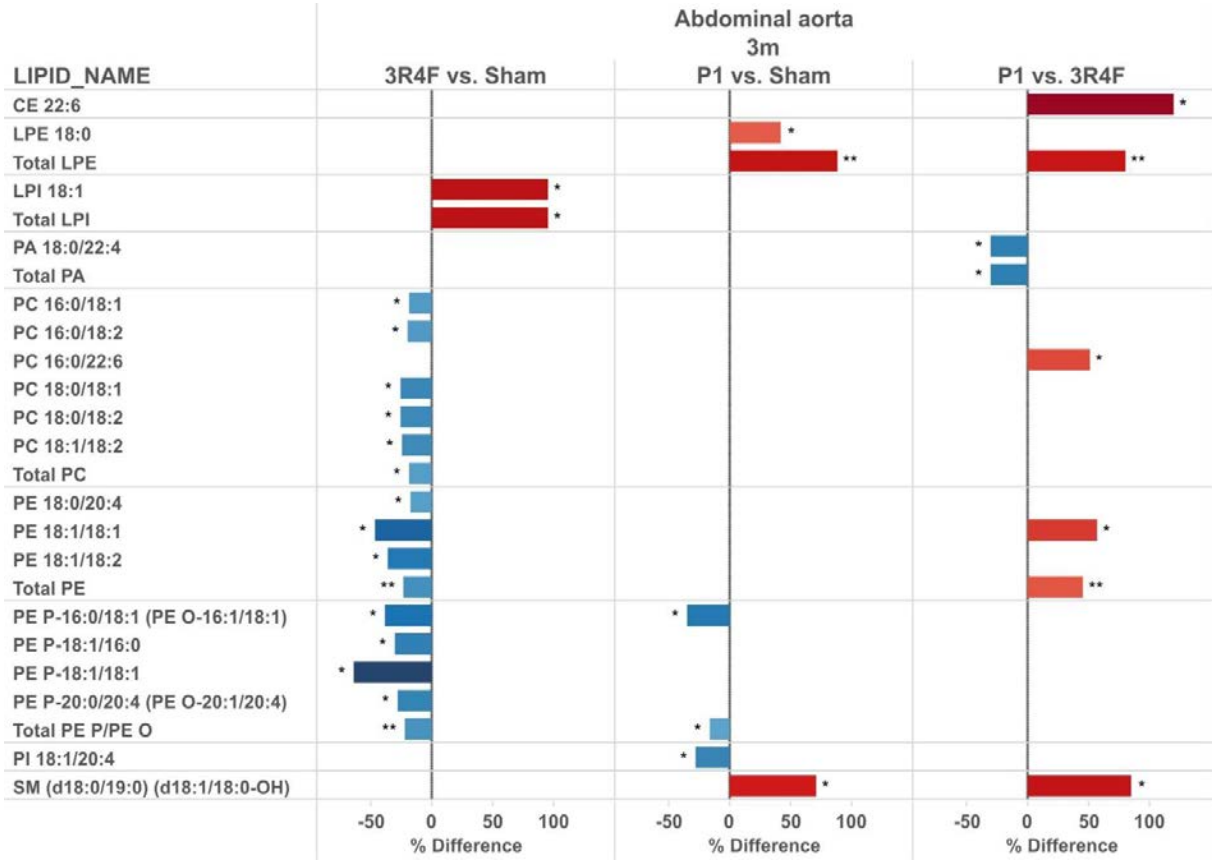



**Figure 14.** Relative percentage differences in abdominal aorta at 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

The relative changes of lipids compared to the 3R4F group are shown in **Figure 15** for ceramides and in **Figure 16** for other lipids. Cessation and switch groups showed increase of ceramides and glycosylated ceramides, and this trend was also seen in the P1 group, although none of the lipids reached statistical significance (results shown in the Tableau file for P1 group). In the switch and cessation groups, several phosphatidylcholines and phosphatidylethanolamines were increased as compared to the 3R4F group.

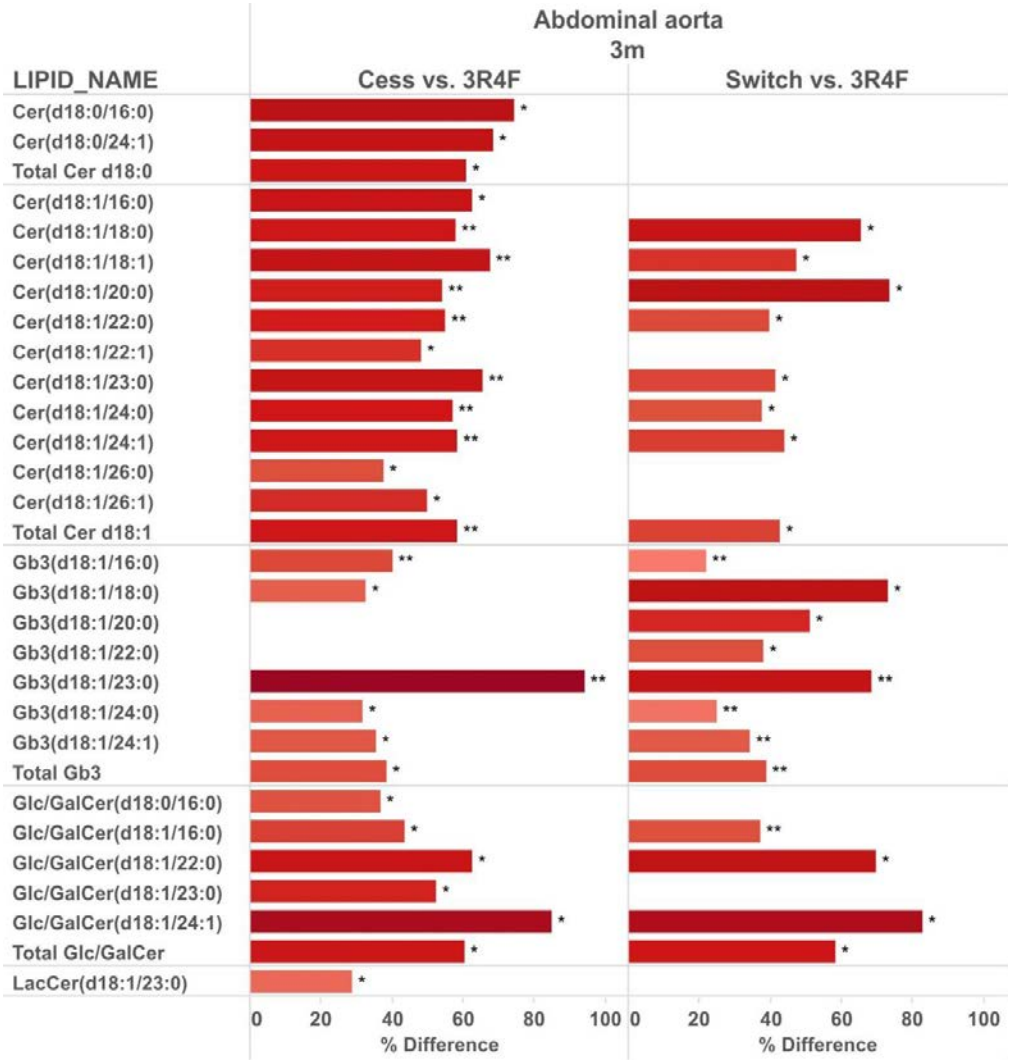



**Figure 15.** Relative percentage differences in abdominal aorta at 3 month time point when compared to the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

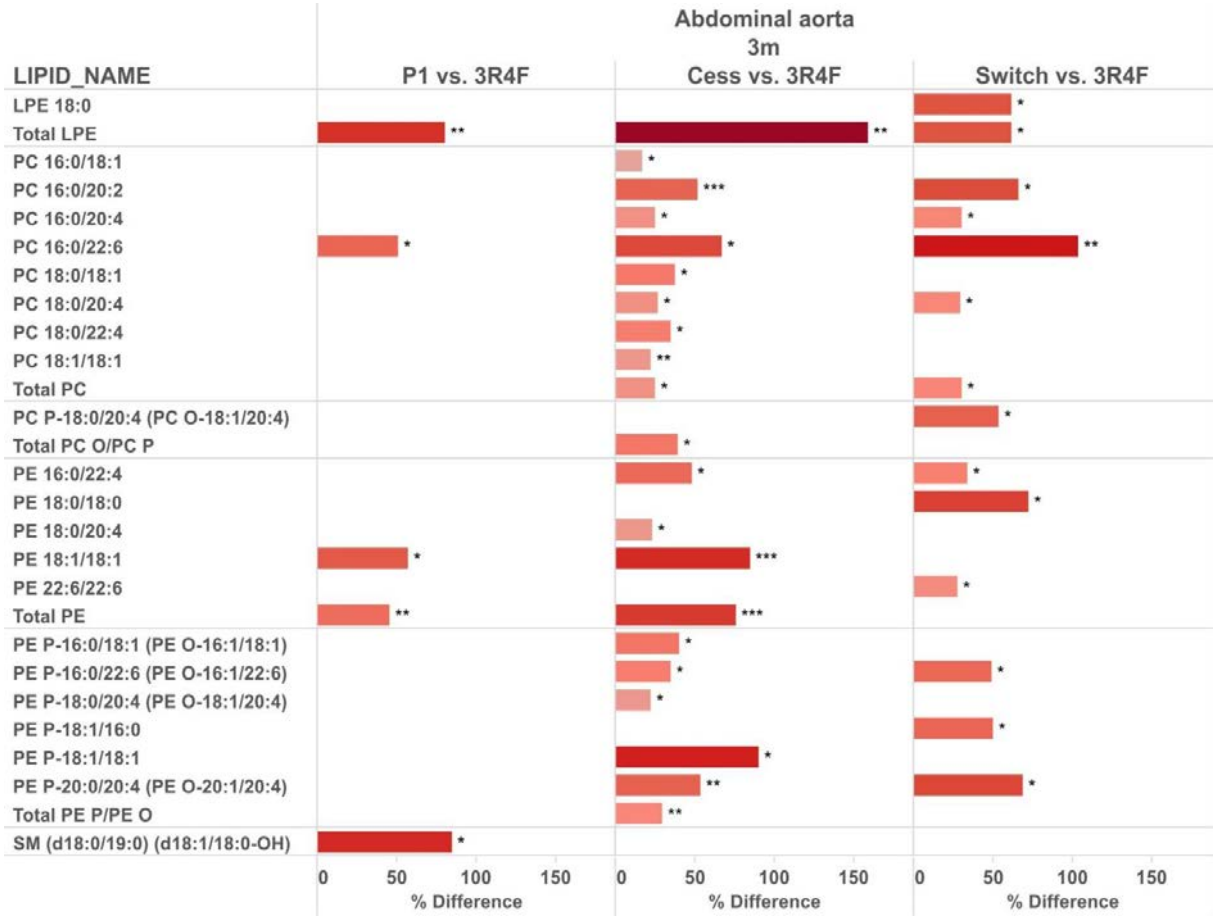



**Figure 16.** Relative percentage differences in abdominal aorta at 3 month time point when compared to the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.3.4 Summary

Consistent with the 2 month results, also at 3 month time point phosphatidylglycerols were increased in the lung samples of the 3R4F group, whereas this was not evident in the P1 group, and the effect was diminished in the cessation and switch groups. Similar to 2 month results the liver showed decreased levels of ceramides and cerebrosides in all groups as compared to the Sham group. Moreover, 3R4F group showed decrease in triacylglycerols and increase in phosphatidylcholines. In abdominal aorta the ceramides and some phospholipids were increased especially in the cessation and switch groups.

## 10.4 Comparisons at 8 month time point

### 10.4.1 Plasma

**Platforms: TAG, CER, FC**

The relative differences for all the groups as compared to the Sham group are shown in **Figure 17**. The results for the cessation group are missing from the figure, because none of the lipids showed statistically significant result as compared to the Sham group. Triacylglycerols were statistically significantly increased in the 3R4F group, and there was also a trend in the P1 group (although not statistically significant), whereas the cessation and switch groups did not show increased levels. From the other lipid classes some ceramides and glycoceramides showed decrease in the 3R4F, P1 and switch groups, although in general these were not the same lipid molecules. Interestingly, Cer(d18:1/18:0) and Glc/GalCer(d18:0/16:0) were decreased the most in the 3R4F group, and this has been observed also in the previous studies (see Summary and Discussion section for more details).

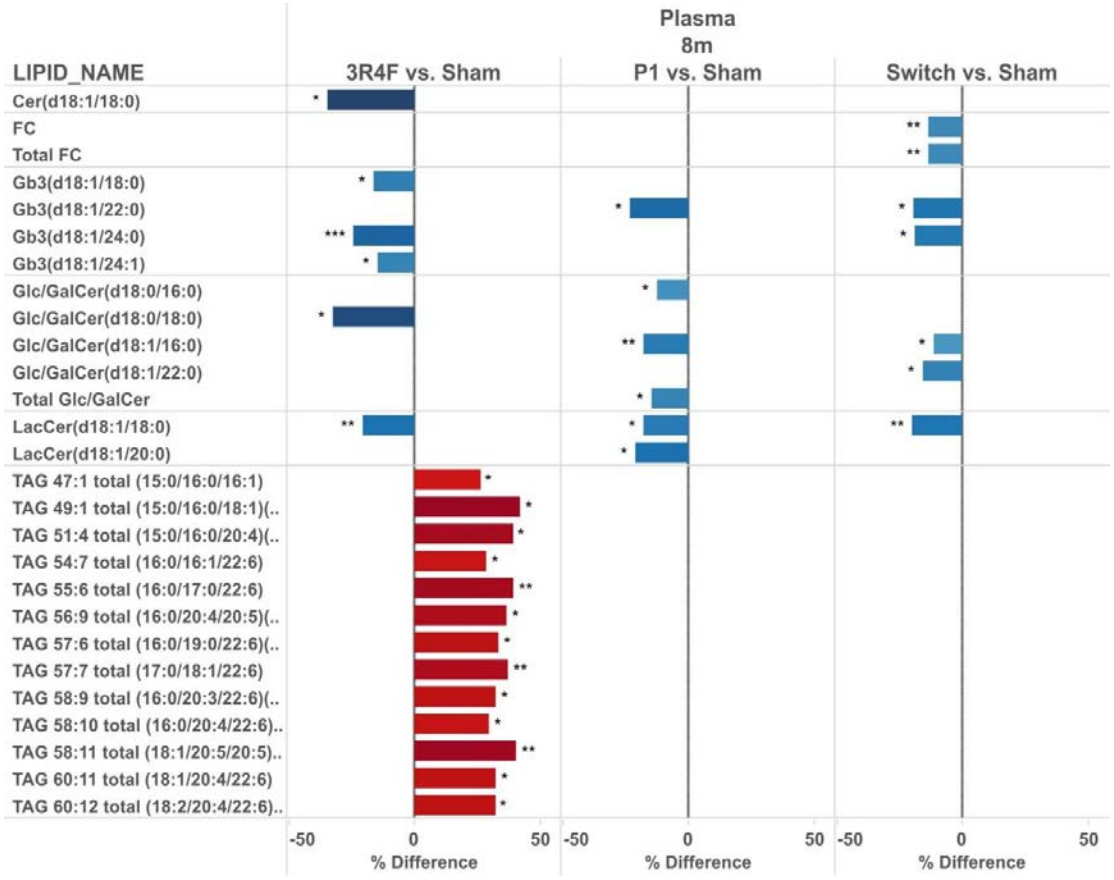



**Figure 17.** Relative percentage differences in plasma at 8 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

The relative differences for all the groups as compared to the 3R4F group are shown in **Figure 18**. The cessation and switch groups showed a clear decrease in triacylglycerols. This trend was also evident in the P1 group, although fewer lipids reached statistical significance. The decrease of ceramides was most obvious in the switch group, although similar trend was observed in the cessation and P1 groups as well.

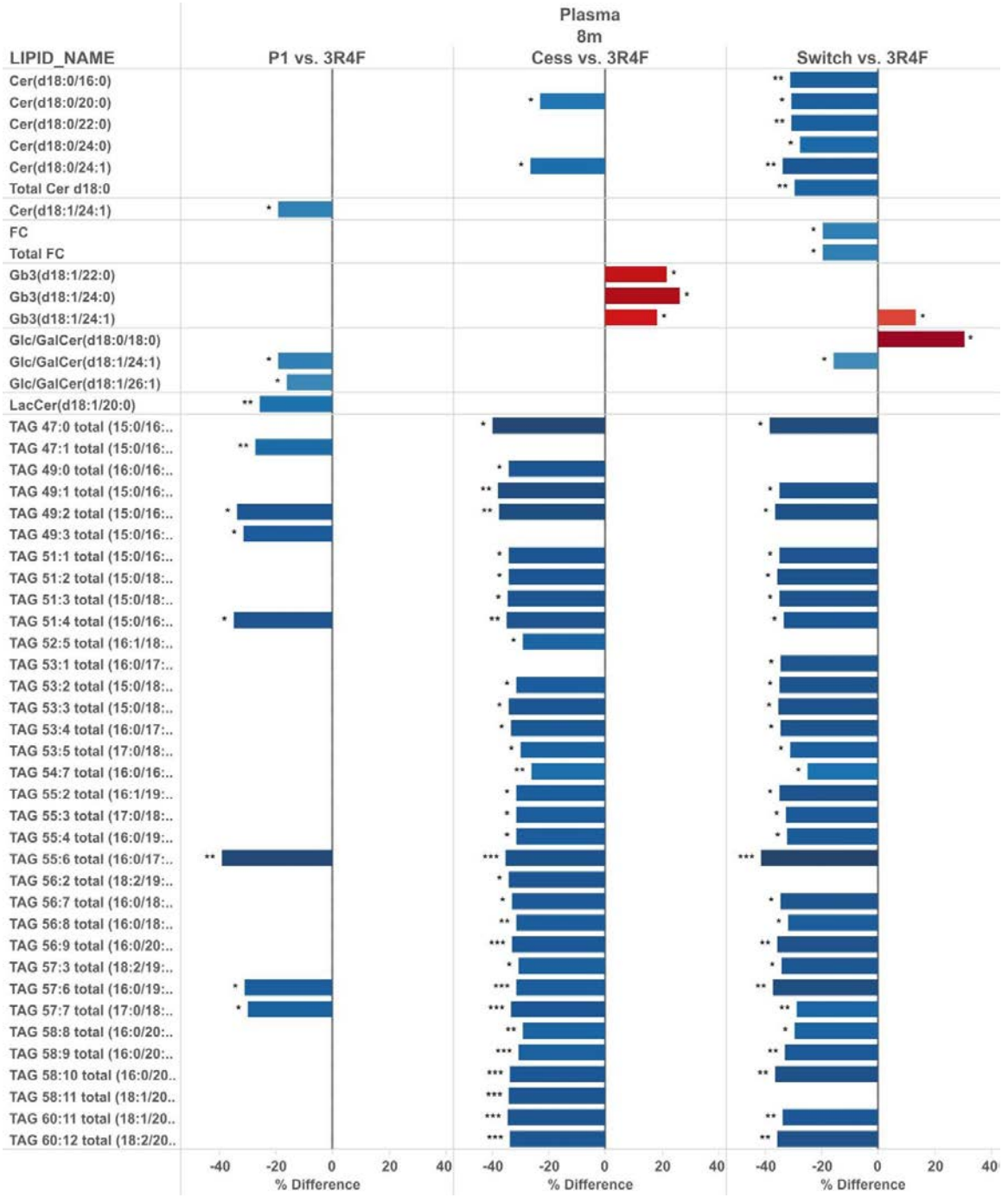



**Figure 18.** Relative percentage differences in plasma at 8 month time point when compared to the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.4.2 Lung

**Platforms: SG, CER**

The relative differences for all the groups as compared to the Sham group are shown for phospholipids in **Figure 20** and for the other lipids in **Figure 21**. Due to high number of significant phospholipids only those showing p<0.001 are shown, and in **Figure 21** all the lipids with p<0.05 are shown. As evident from the figures, only in the 3R4F group there was a strong increase for phospholipids, and consistent with the previous time points the highest increase was observed for PG(18:2/20:4) (detailed data for this lipid is shown in **Figure 19**). Ceramides and cerebrosides were also increased in the 3R4F group, while this was not the case for any other group. However, in the switch group many ceramides and glycosylated ceramides showed a decreasing trend as compared to the Sham group.

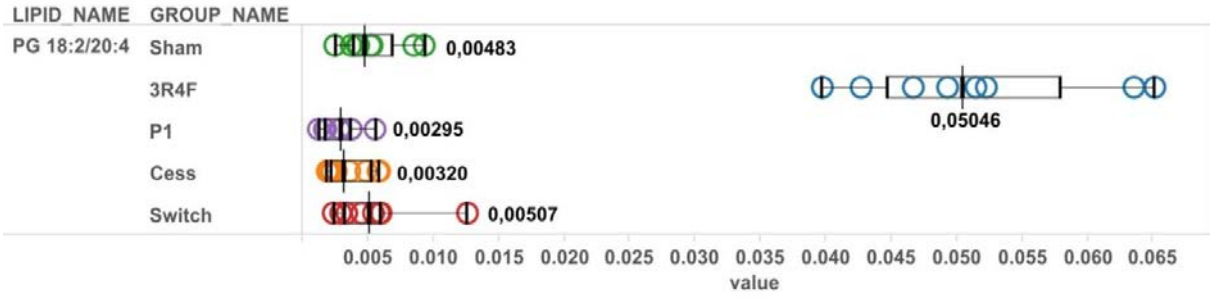



**Figure 19.** Concentration of PG 18:2/20:4 in lung at 8 month time point.

The relative differences for all the groups as compared to the 3R4F group are shown for phospholipids in **Figure 22** and for the other lipids in **Figure 23**. Due to high number of significant lipids in these figures only those showing p<0.001 are shown. There was an overall strong decreasing trend for phospholipids in all groups, i.e. P1, cessation and switch against the 3R4F group. The strongest decrease was again shown for the phosphatidylglycerols. The downregulation of ceramides was observed in the P1 and switch groups, although some ceramides showed the opposite trend, and e.g. Cer(d18:1/18:1) was increased in all groups (result shown in Tableau, but not in **Figure 23**).

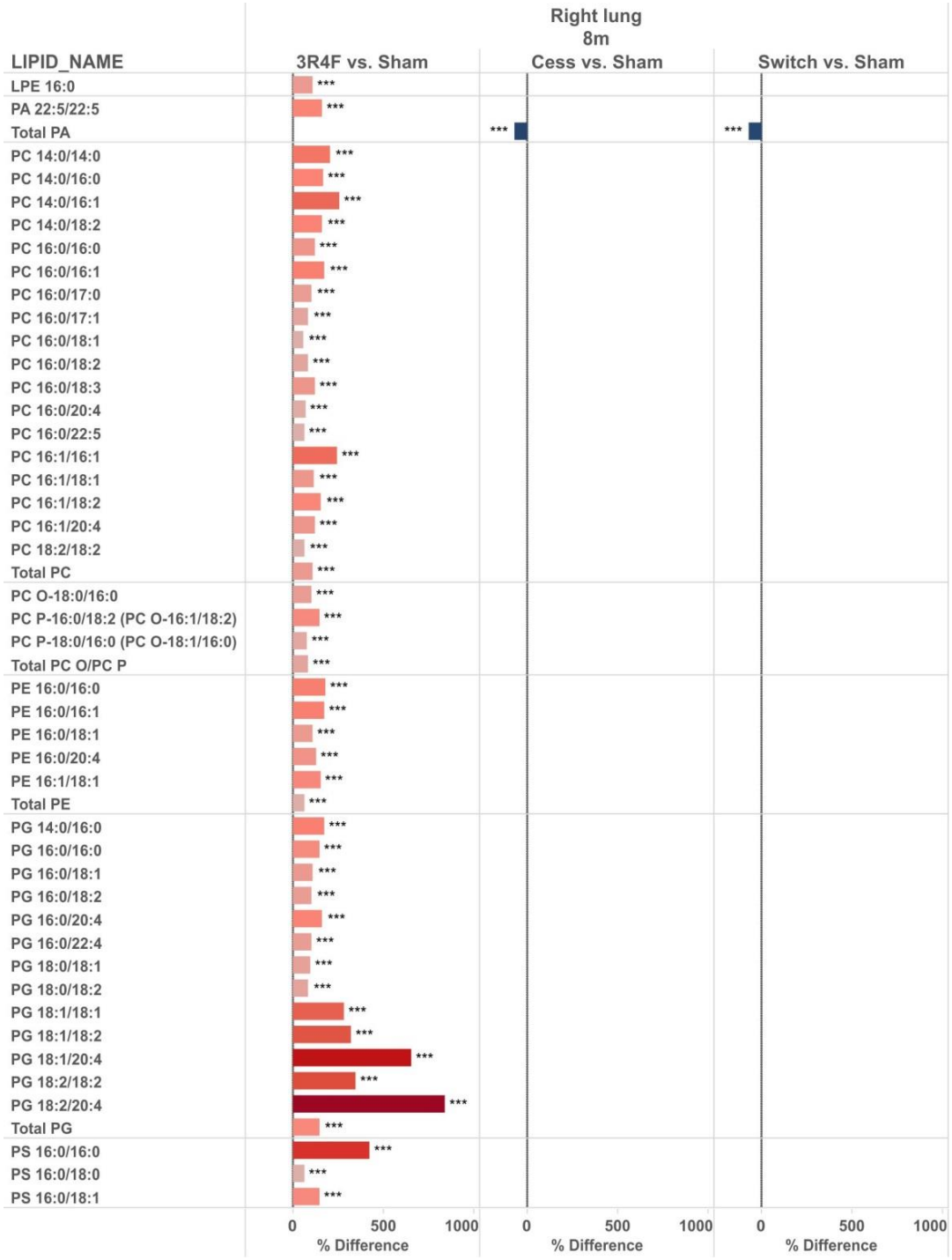



**Figure 20.** Relative percentage differences for phospholipids in lung at 8 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

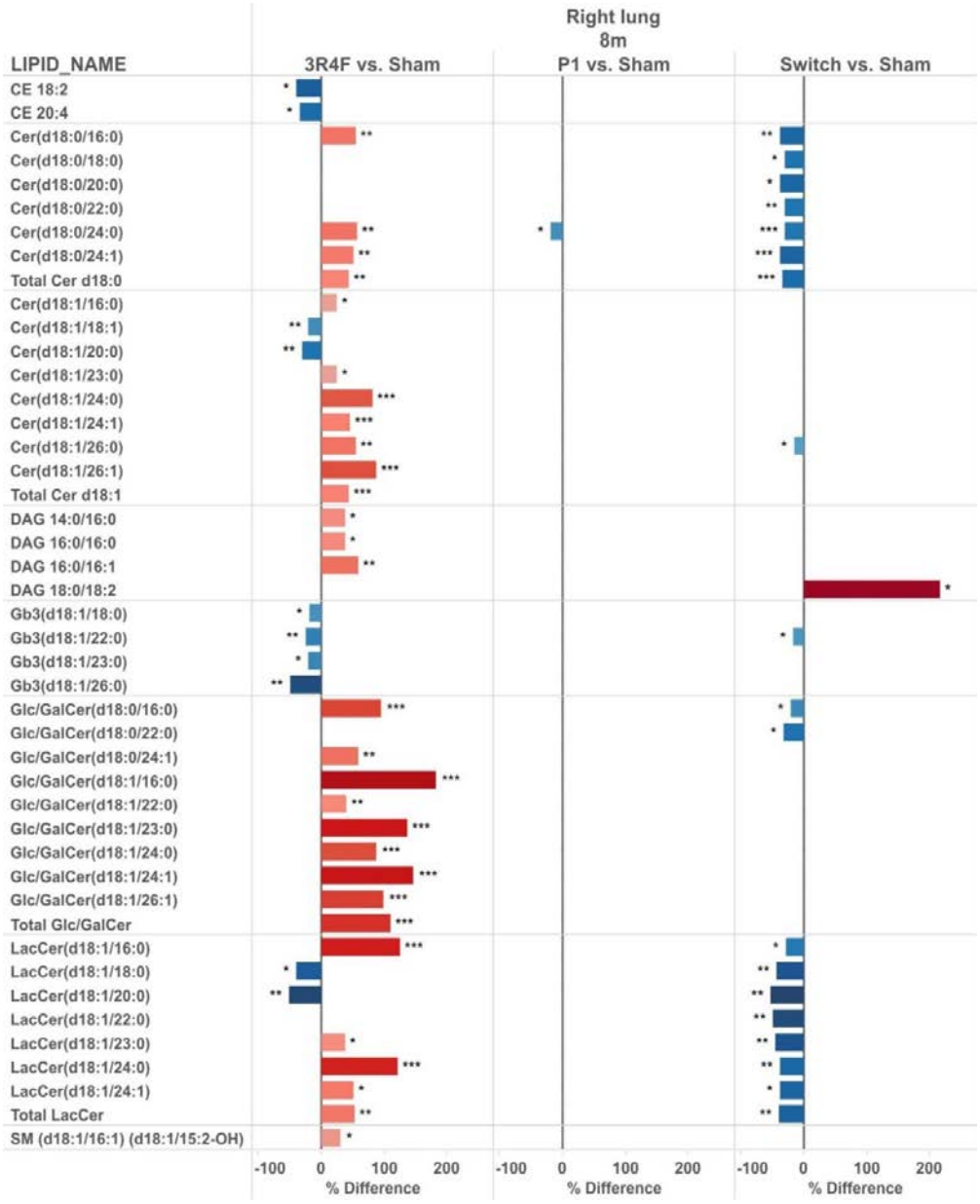



**Figure 21.** Relative percentage differences in lung for other than phospholipids at 8 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

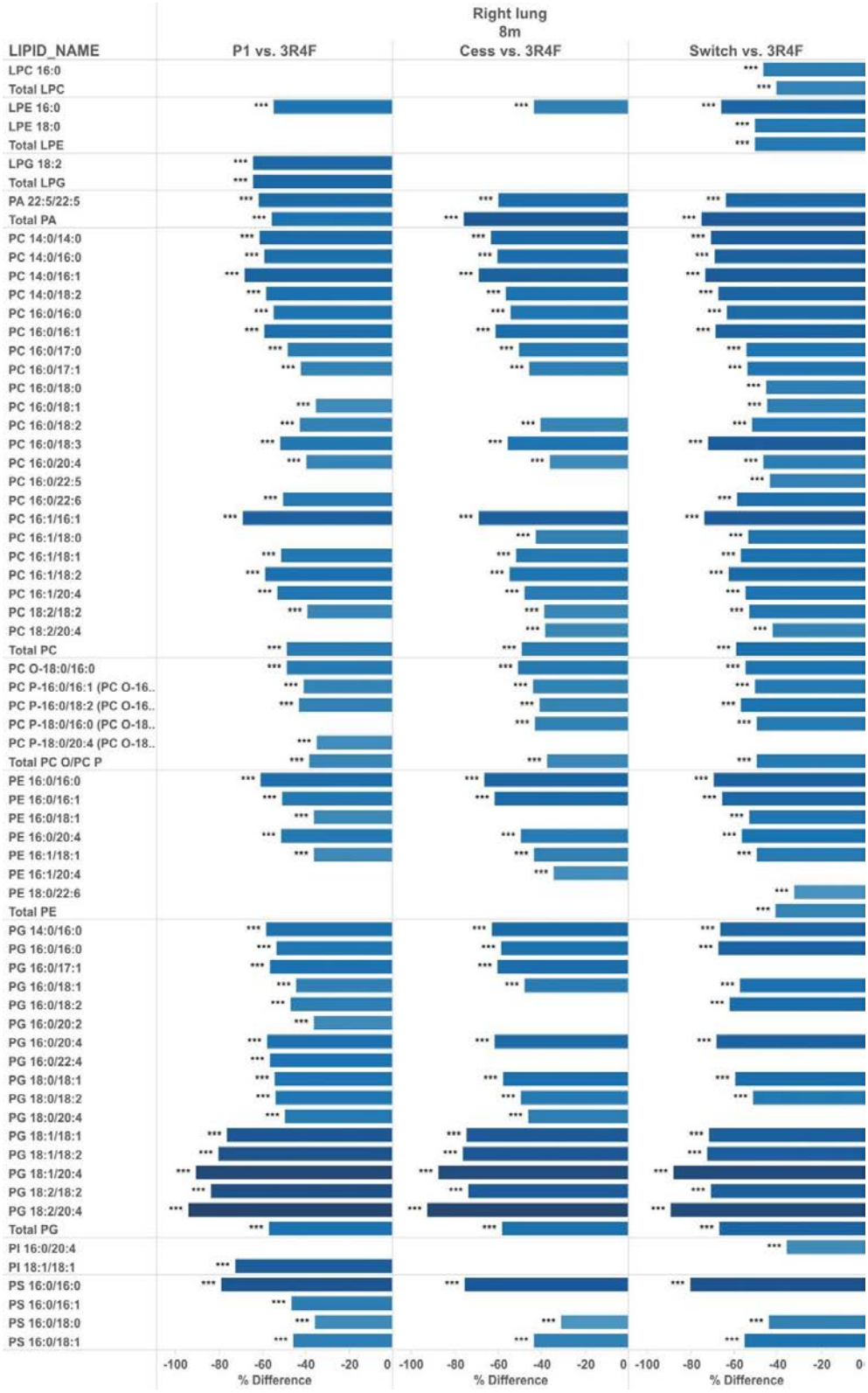



**Figure 22.** Relative percentage differences for phospholipids in lung at 8 month time point when compared to the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

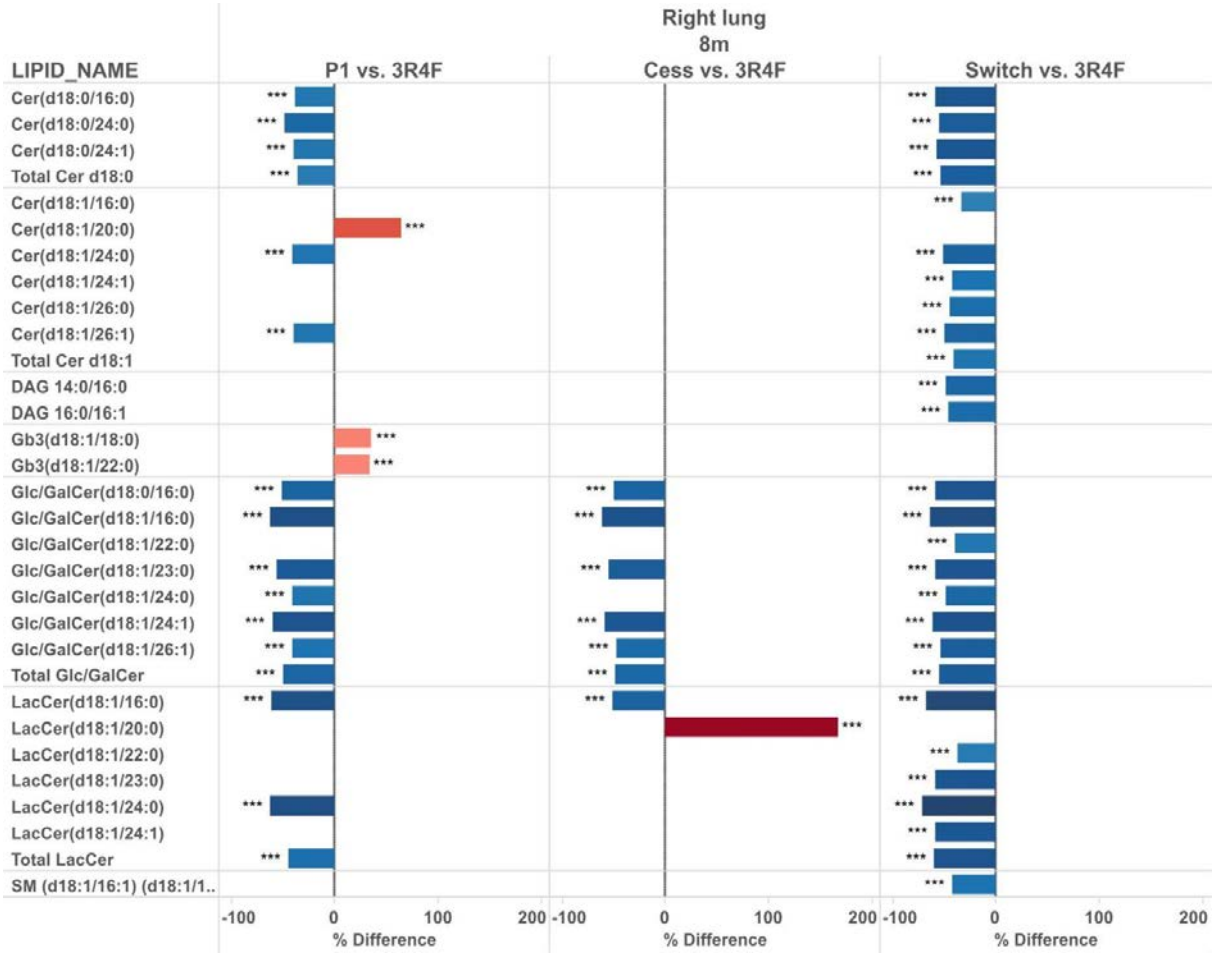



**Figure 23.** Relative percentage differences in plasma for other than phospholipids at 8 month time point when the comparison is made against the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.4.3 Liver

**Platforms: SG, TAG, CER**

The relative difference of ceramides for all the groups as compared to the Sham group are shown in **Figure 24** and for other lipids in **Figure 26** (only p<0.01 shown in this figure). Similar to the findings at 2 and 3 month time point, there was an overall decrease in the ceramides and cerebrosides. The changes were prominent in all the other groups than in the 3R4F group. The same trend was observed also for many other lipids, where the P1, cessation and switch groups showed more changes than the P1 group. When comparing the ceramide results to the 3R4F group, the most notable changes was the decrease of lactosylceramides in all groups and decrease of ceramides in the cessation and switch groups (

**Figure 25**). For other lipids there was a decrease as compared to the 3R4F group for phospholipids and cholesteryl esters and increase of certain diacylglycerols, as shown in **Figure 27**.

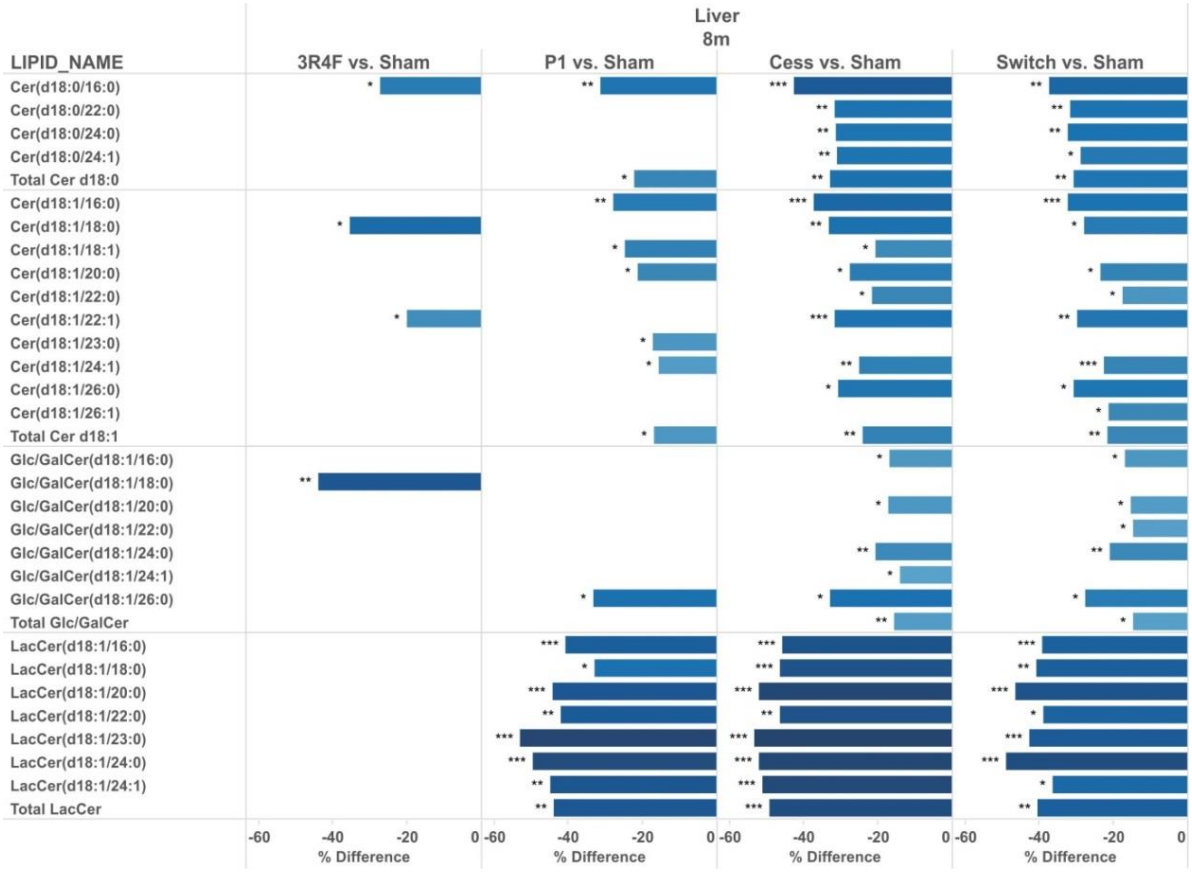



**Figure 24.** Relative percentage differences of ceramides in liver at 8 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

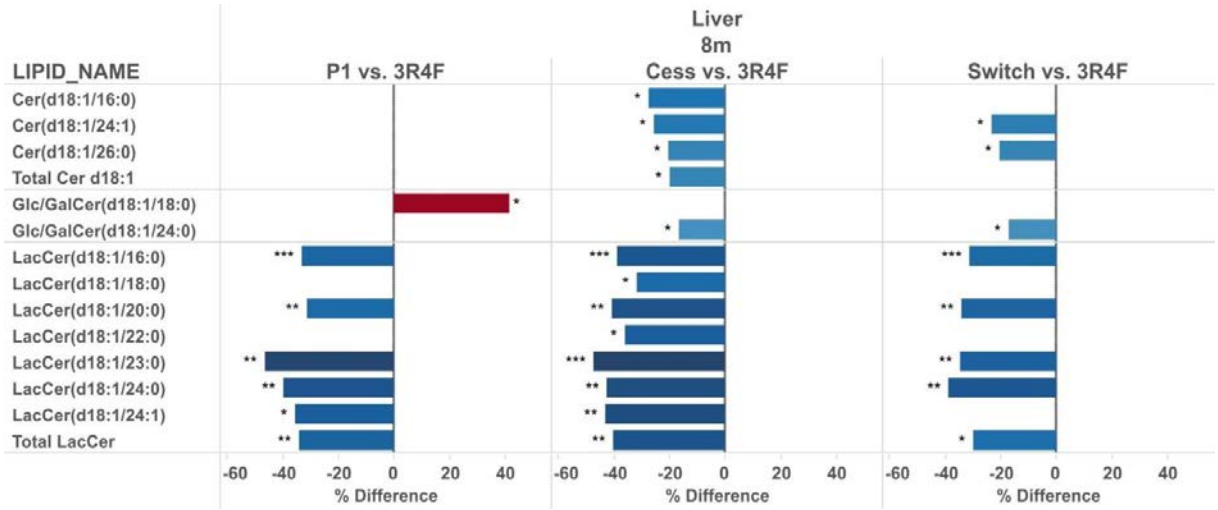



**Figure 25.** Relative percentage differences of ceramides in liver at 8 month time point when compared to the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

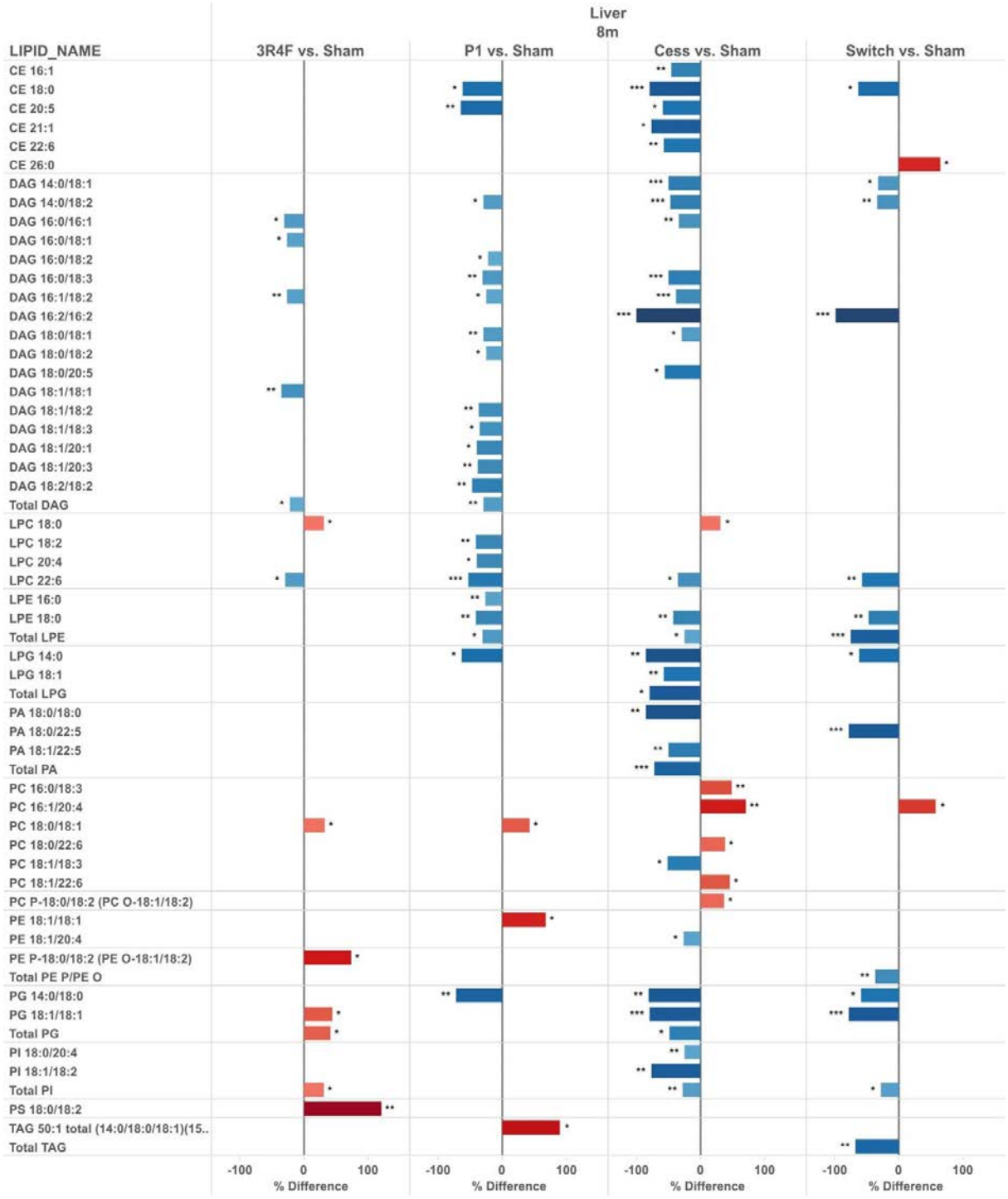



**Figure 26.** Relative percentage differences in liver at 8 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

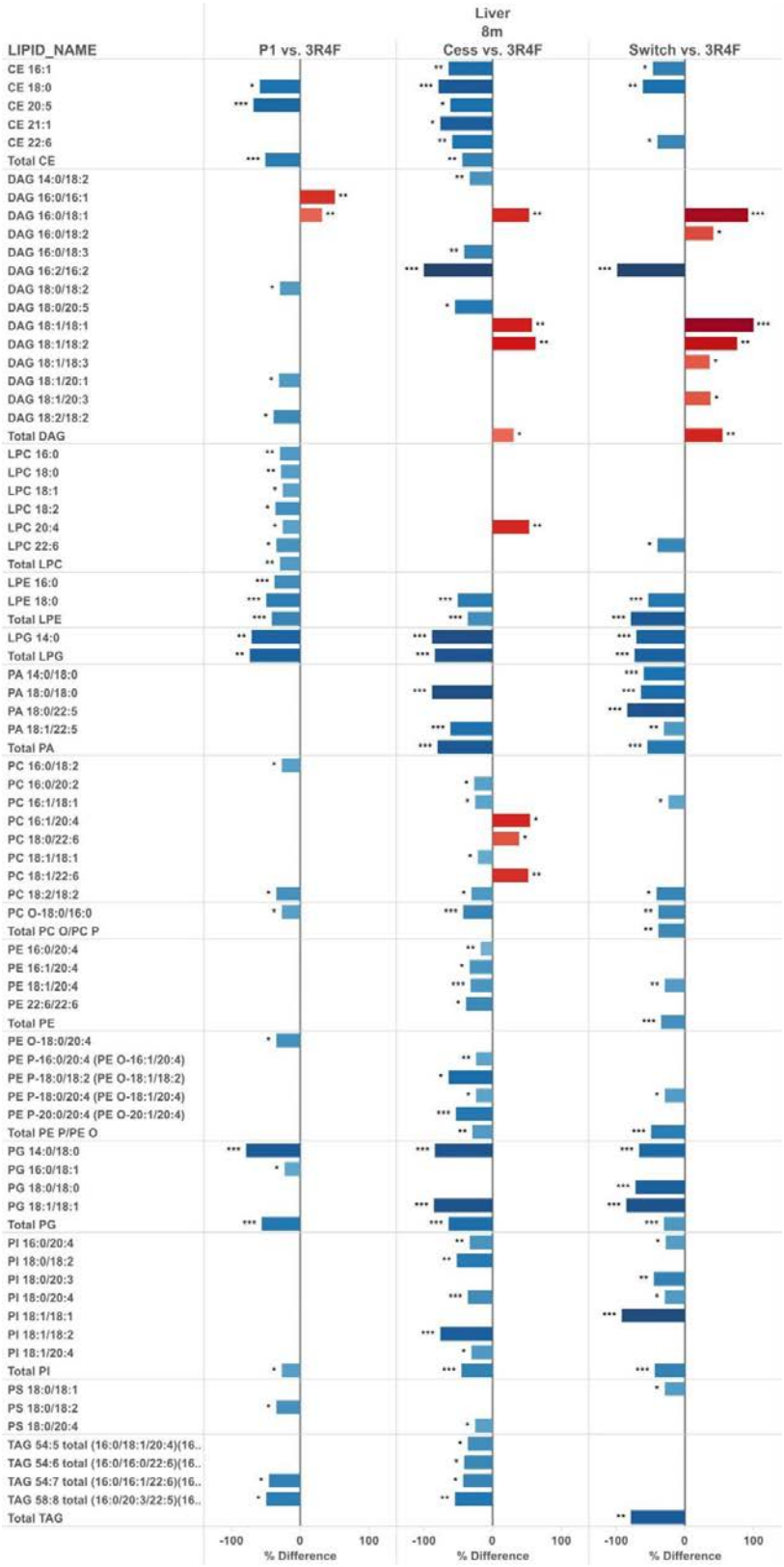



**Figure 27.** Relative percentage differences in liver at 8 month time point when compared to the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.4.4 Abdominal aorta

**Platforms: SG, CER, FC**

The relative difference of ceramides for all the groups as compared to the Sham group are shown in **Figure 28**. Glycosylated ceramides did not show any changes, and there were no statistically significant changes in the P1 group at all. The most dramatic changes were observed in the cessation group. The results for other lipids are shown in **Figure 41**. P1 group showed an increase in diacylglycerols, but no other major differences were observed.

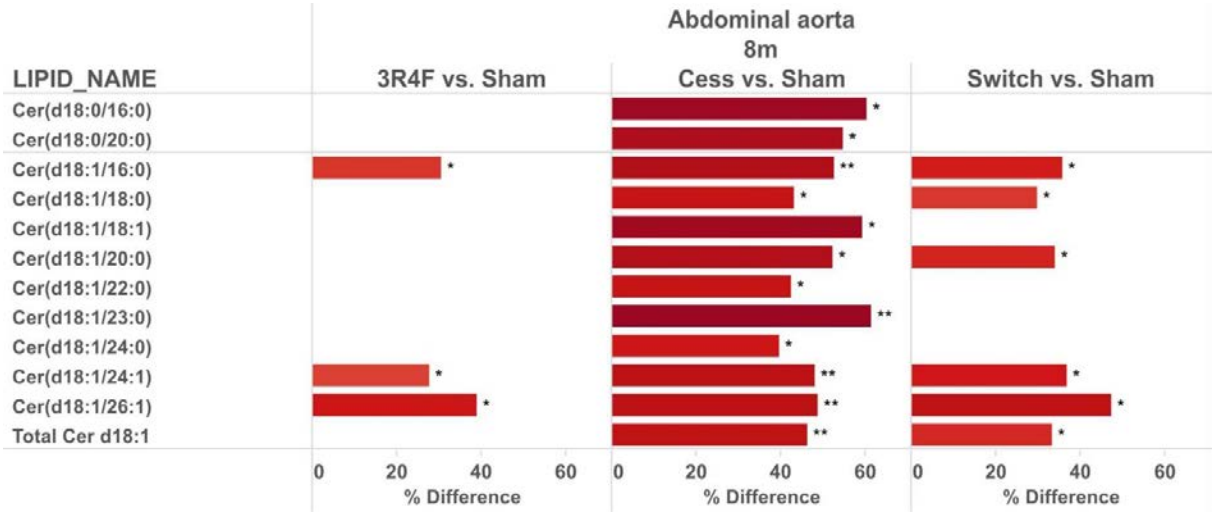



**Figure 28.** Relative percentage differences in abdominal aorta at 8 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

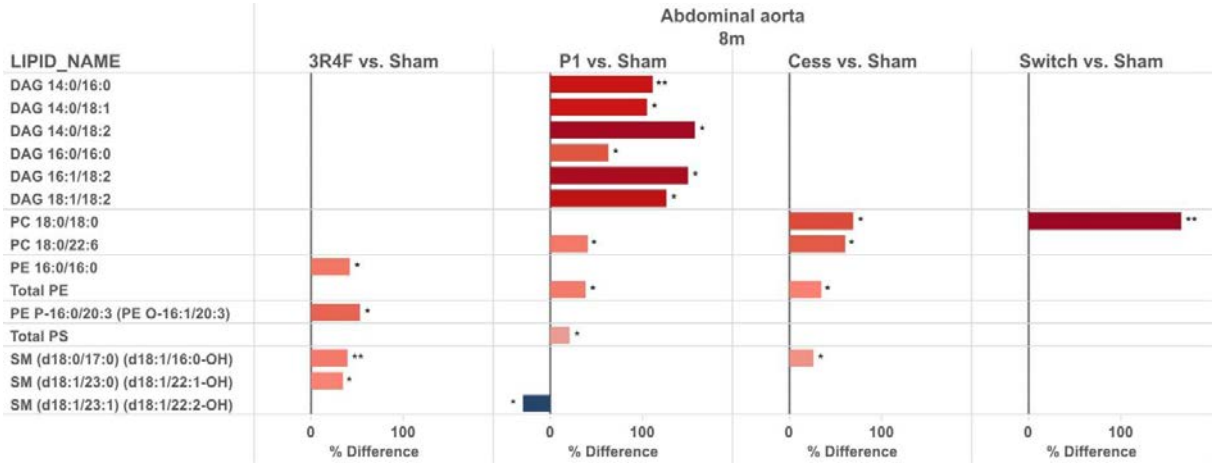



**Figure 29.** Relative percentage differences in abdominal aorta at 8 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

When performing the same comparison against the 3R4F group, the diacylglycerols were increased and lysophosphatidylcholines and sphingomyelins were decreased in the P1 group, as shown in **Figure 30**.

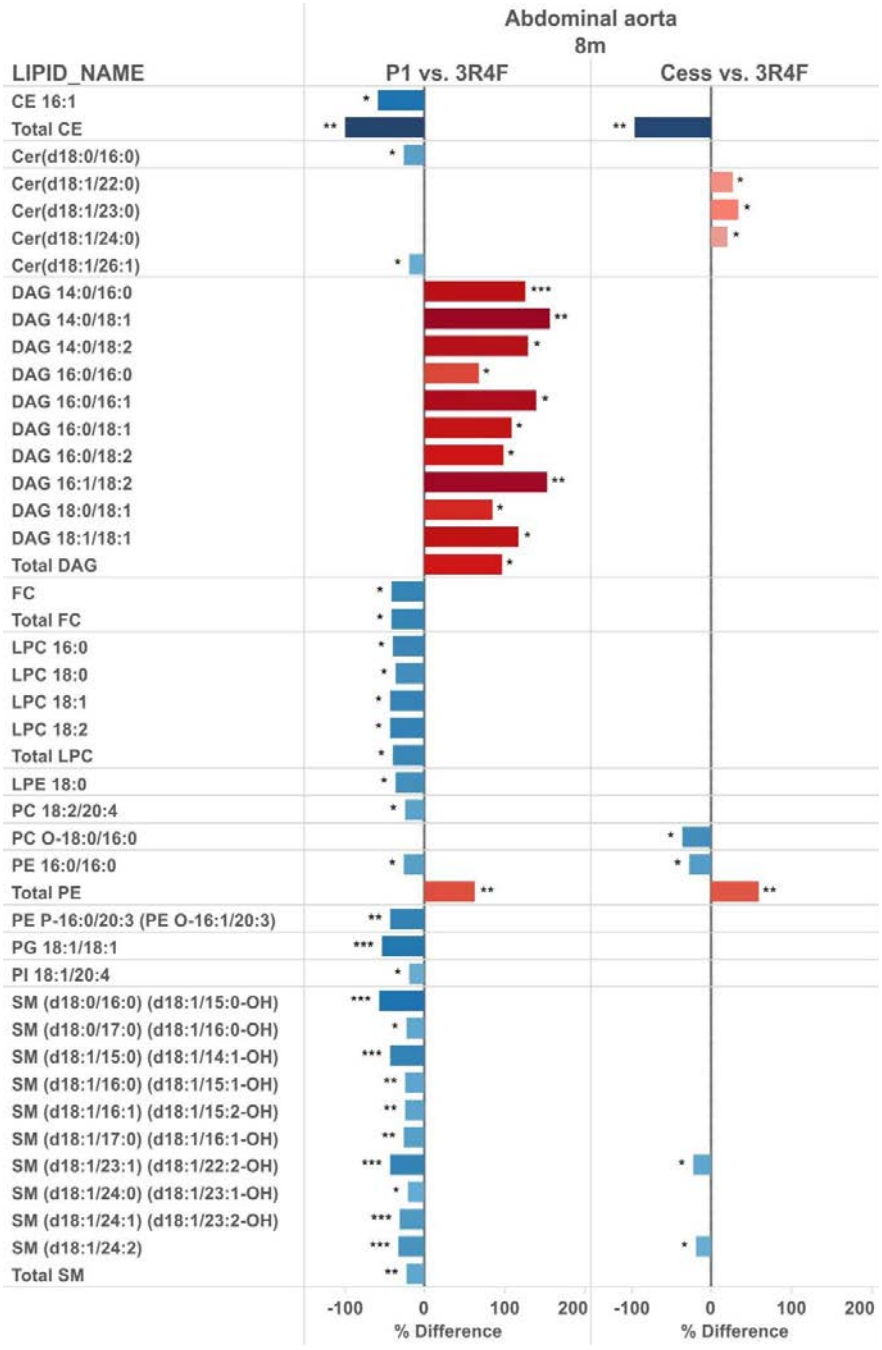



**Figure 30.** Relative percentage differences in abdominal aorta at 8 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.4.5 Aortic arch

**Platforms: SG, CER, FC**

The relative difference of ceramides for all the groups as compared to the Sham group are shown in **Figure 31**. Ceramides and glycosylated ceramides were increased in the 3R4F group, but no statistically significant change was observed in the P1 or switch groups, and only a few lipids were statistically significantly increased in the cessation group.

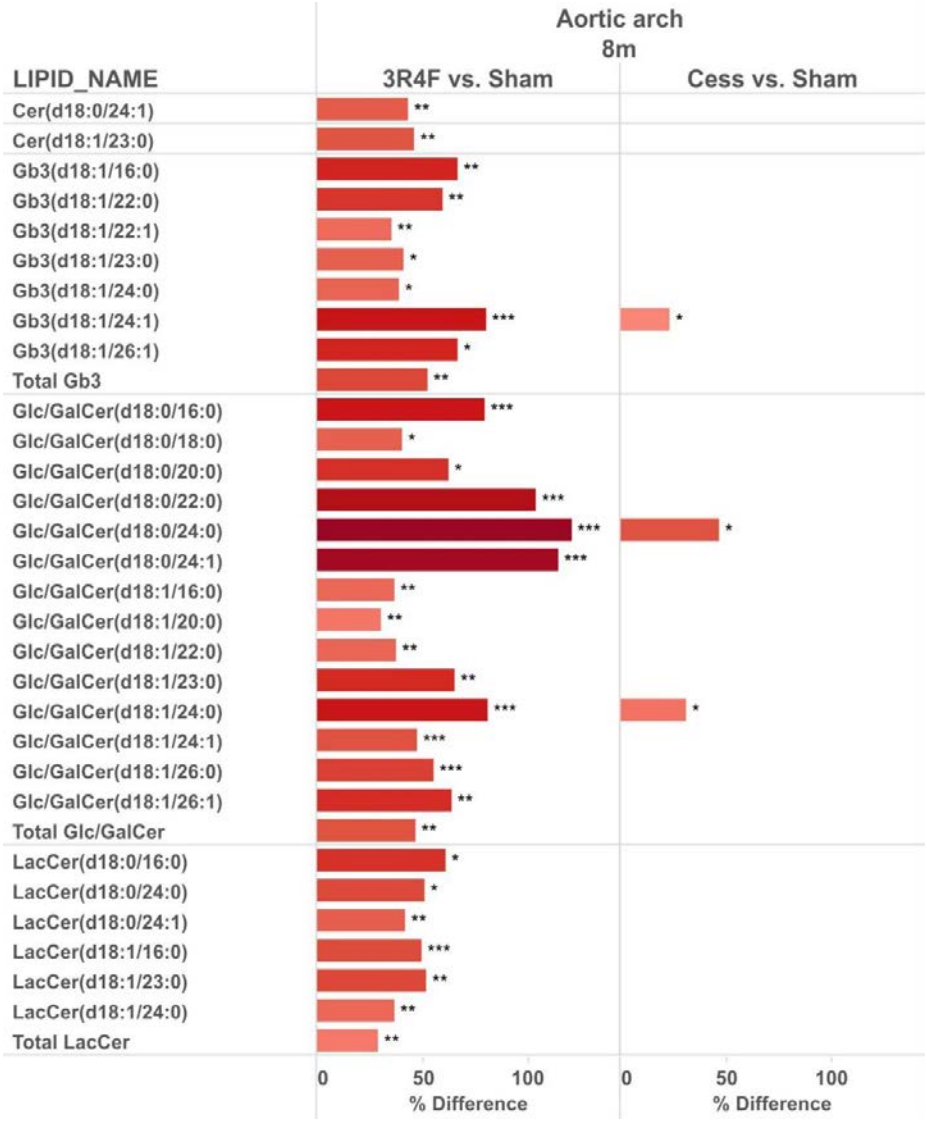



**Figure 31.** Relative percentage difference of ceramides in aortic arch at 8 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

When the comparison is against the 3R4F group, there is a clear decrease in ceramides and cerebrosides in all groups, with the most prominent change observable in the P1 group (**Figure 32**).

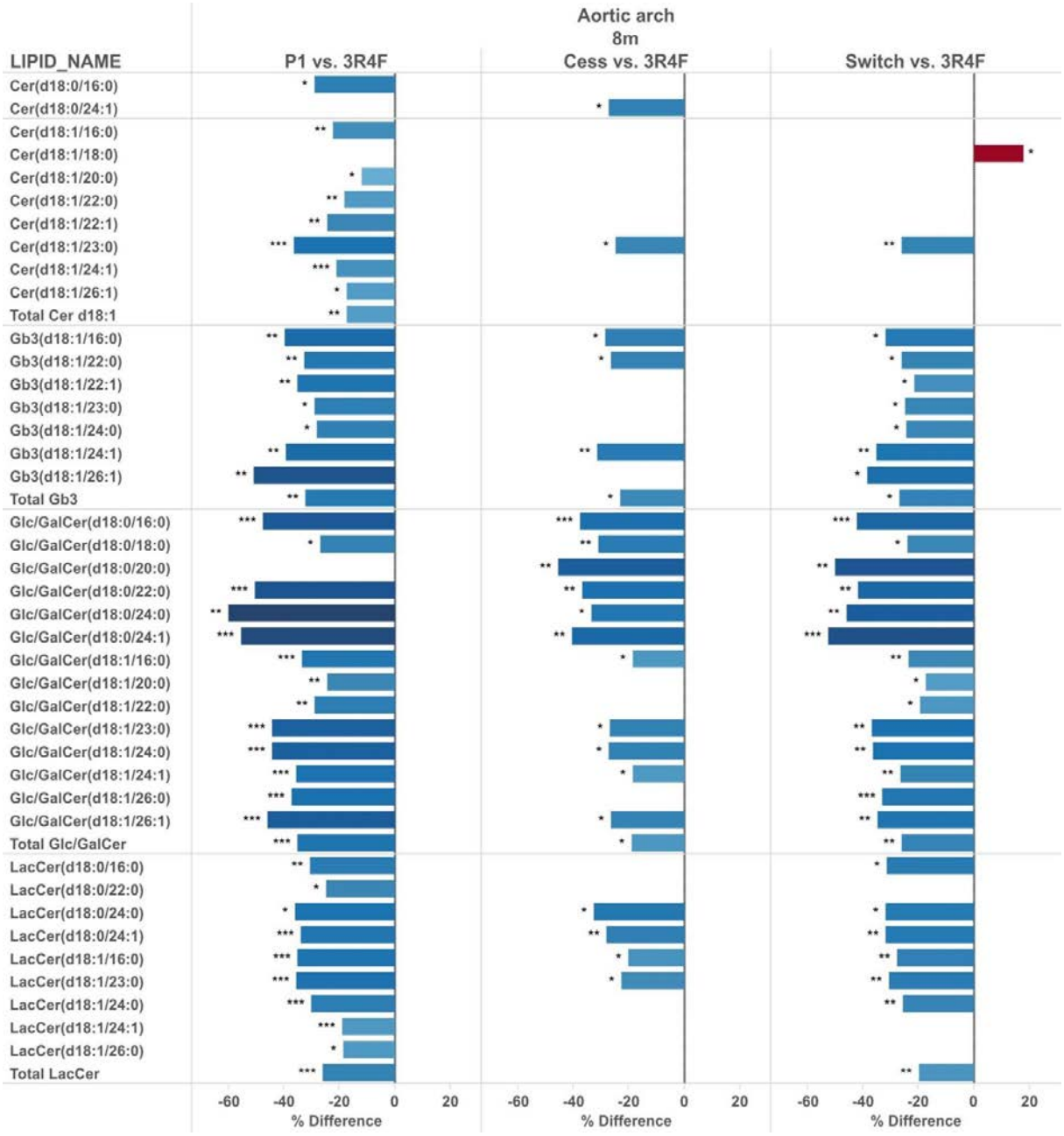



**Figure 32.** Relative percentage differences of ceramides in aortic arch at 8 month time point when compared to the 3R4F group. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

For other lipids than ceramides there were dramatic differences mainly in the 3R4F group. Cholesteryl esters, free cholesterol, phospholipids and sphingomyelins were all increased in the 3R4F group as compared to the Sham and other groups. The comparison against the Sham as well as the 3R4F groups are shown in **Figure 46** (only p<0.01 are shown).

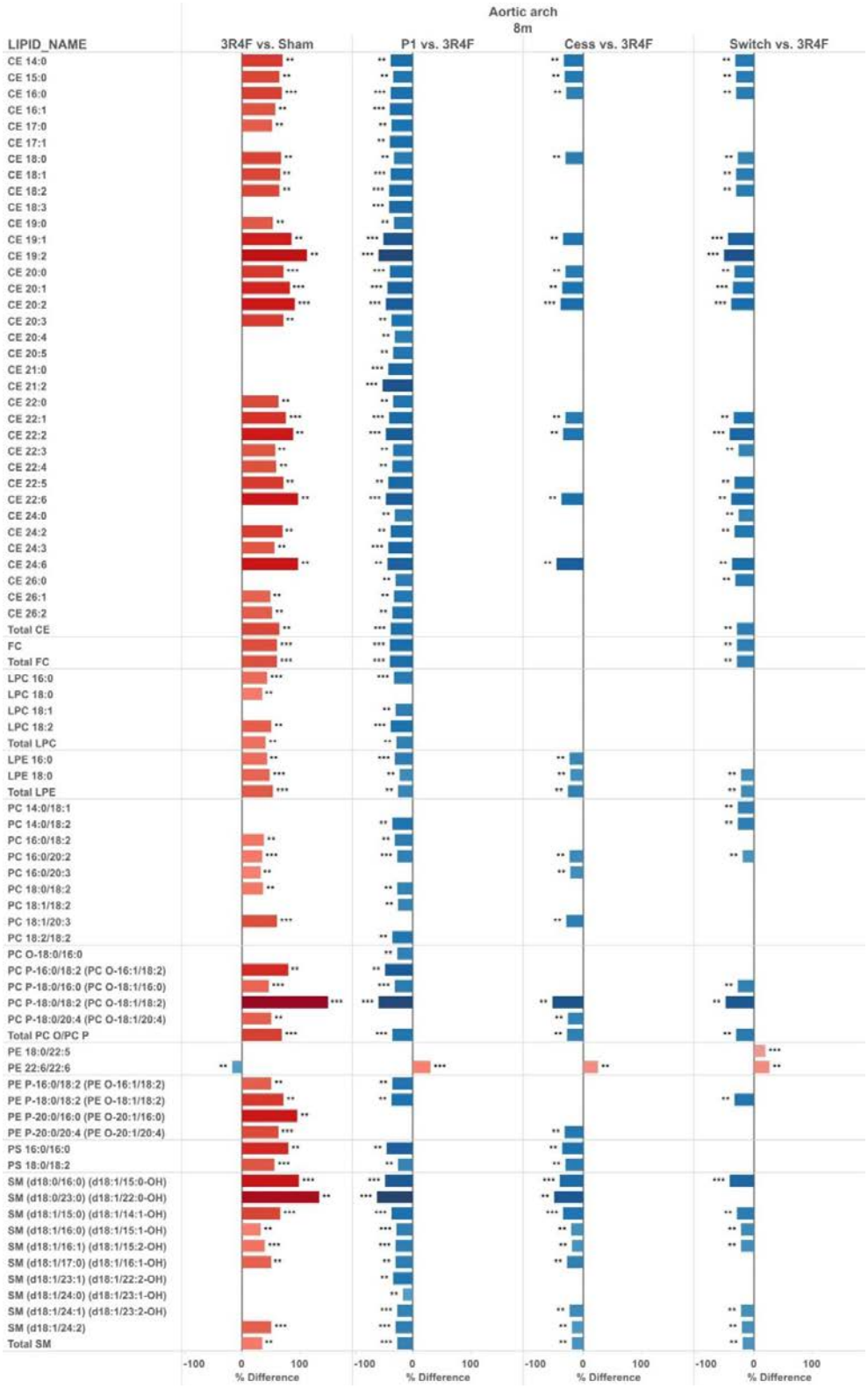



**Figure 33.** Relative percentage differences in aortic arch at 8 month time point when compared to the Sham and 3R4F groups. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.4.6 Summary

In summary, in plasma, lung and aortic arch the 3R4F group was showing clearly different lipidomic profiles as compared to the other groups. In plasma, triacyglycerols were increased, and in lung as well as aortic arch the glycosylated ceramides and phospholipids were increased in the 3R4F group as compared to the Sham group. In aortic arch also cholesteryl esters showed dramatic increase.

Liver was an exception compared to other tissues, as in all other groups than in the 3R4F group there was a decrease of ceramides and glycosylated ceramides, and also the lipids from shotgun analysis showed least changes in the 3R4F group. The results from abdominal aorta samples were surprising in the sense that in that tissue the cessation group showed the strongest changes for ceramides and the P1 group for diacylglycerols.

## 10.5 Pairwise group comparisons: cessation against 3R4F at 2 months

### 10.5.1 Lung

**Platforms: SG, CER**

When the cessation time points (3 and 8 months) were compared to the 3R4F group at 2 month, there was a large decrease of several lipid classes in the lung. Ceramides and cerebrosides **(Figure 34)**, phospholipids **(Figure 35)** and especially phosphatidylglycerols **(Figure 36)** were all decreased, and in general, the decreasing trend at 8 month was stronger than at 3 month time point.

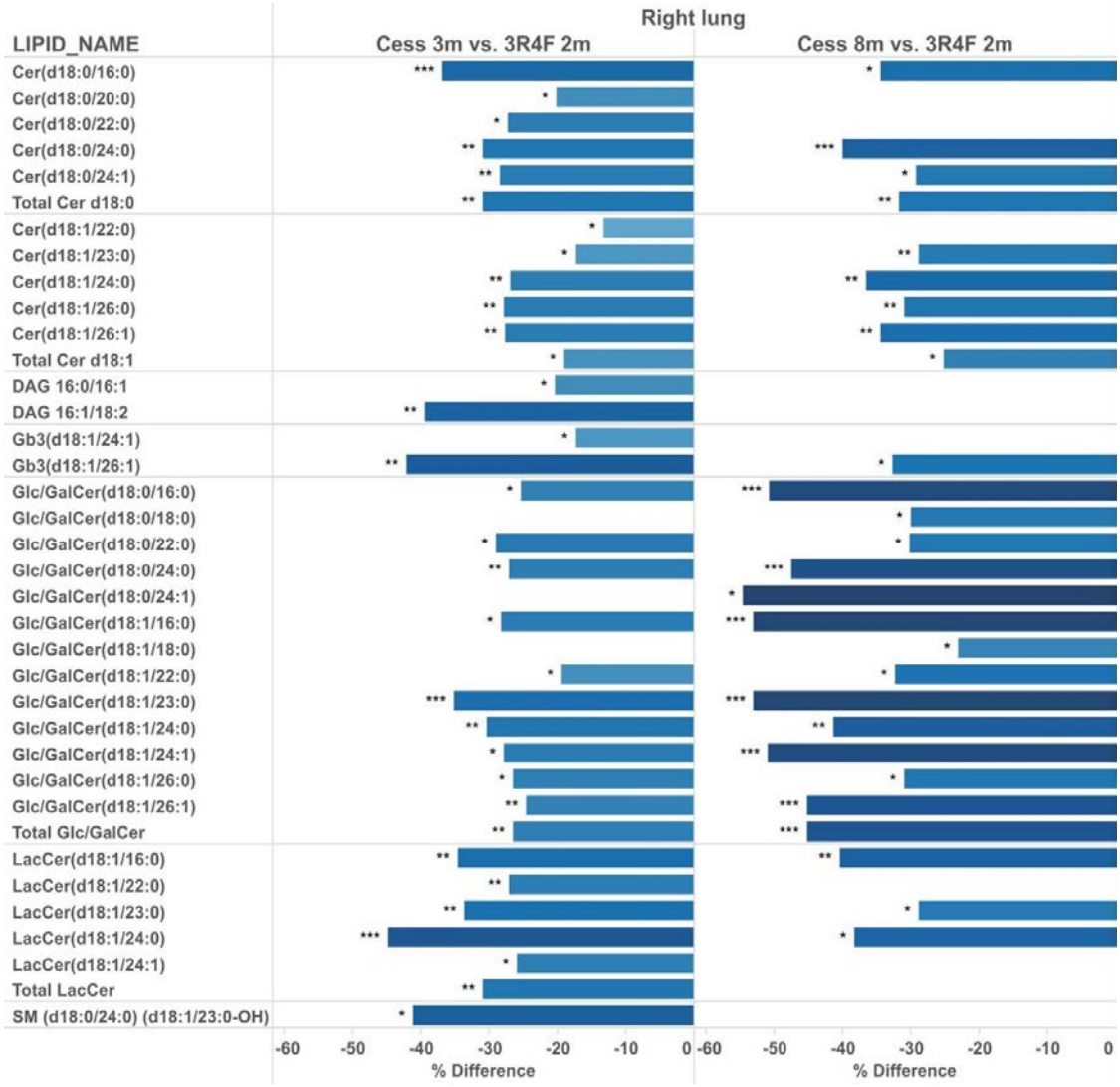



**Figure 34.** Relative percentage differences of sphingolipids and diacylglycerols in lung in the cessation group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

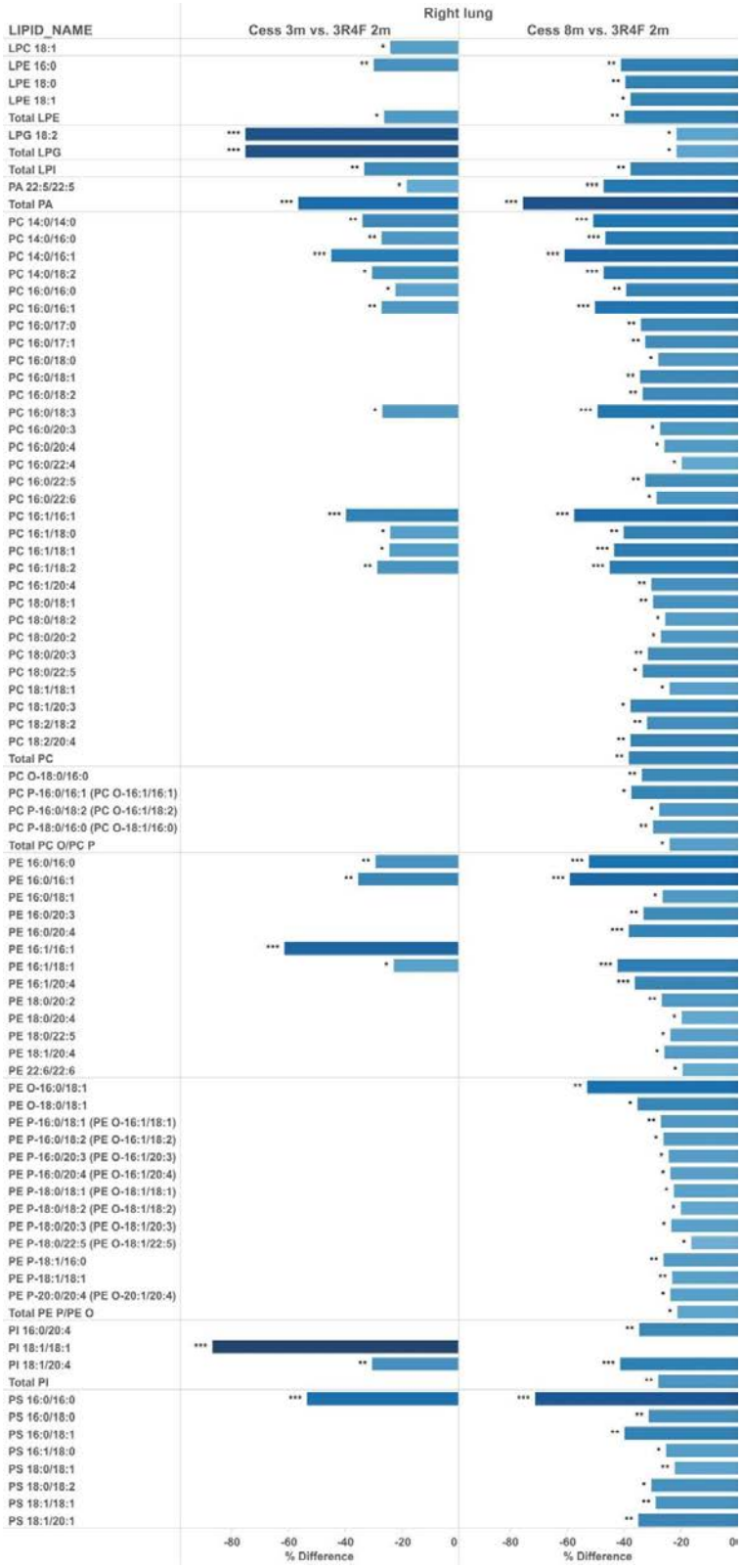



**Figure 35.** Relative percentage differences of phospholipids in lung in the cessation group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

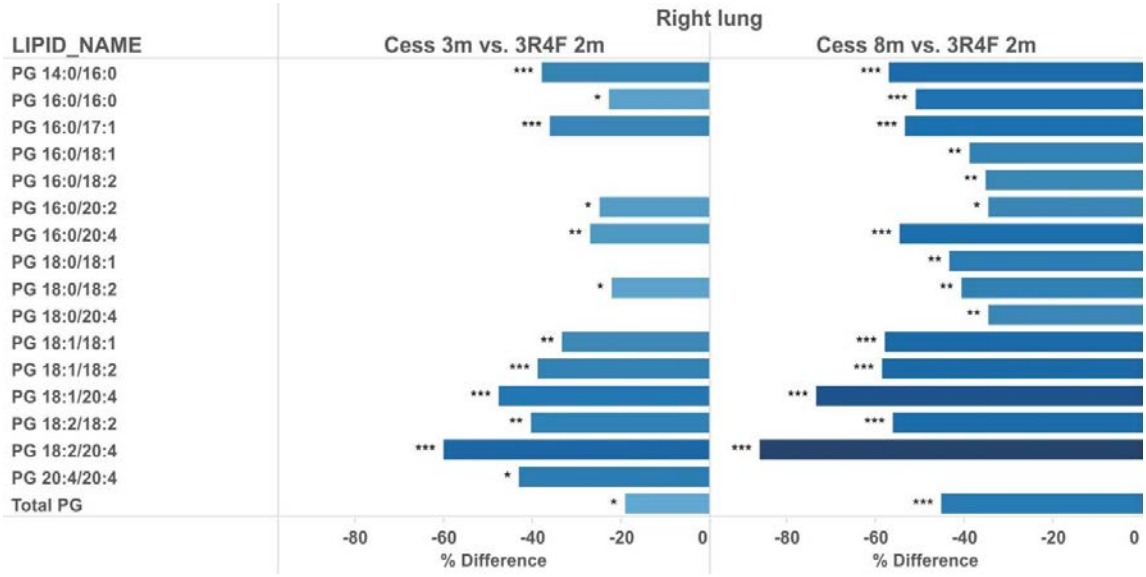



**Figure 36.** Relative percentage differences of phosphatidylglycerols in lung in the cessation group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.5.2 Liver

**Platforms: SG, TAG, CER**

When the cessation time points (3 and 8 months) were compared to the 3R4F group at 2 month time point, most of the ceramides were decreased at 3 month time point (**Figure 37**). This was apparent also for the d18:0 dihydroceramides at 8 month time point, but the cerebrosides and globotriasoylceramides were increased. Considering the other lipids than ceramides, the most notable changes were the increase of diacylglycerols and decrease of cholesteryl esters at both time points and the increase of triacylglycerols at 3 months (**Figure 38**, in this figure only p<0.01 are shown).

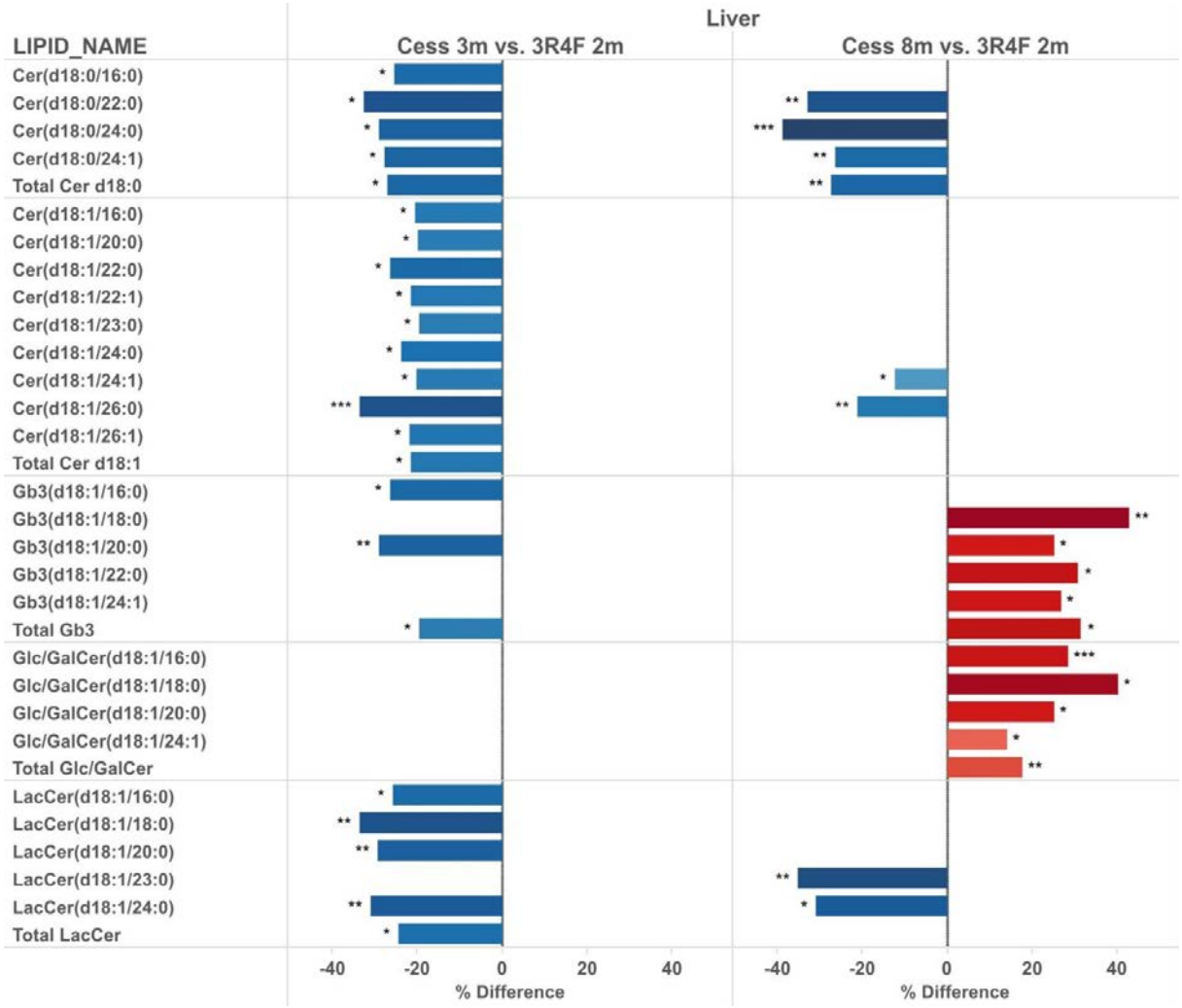



**Figure 37.** Relative percentage differences of ceramides in liver in the cessation group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

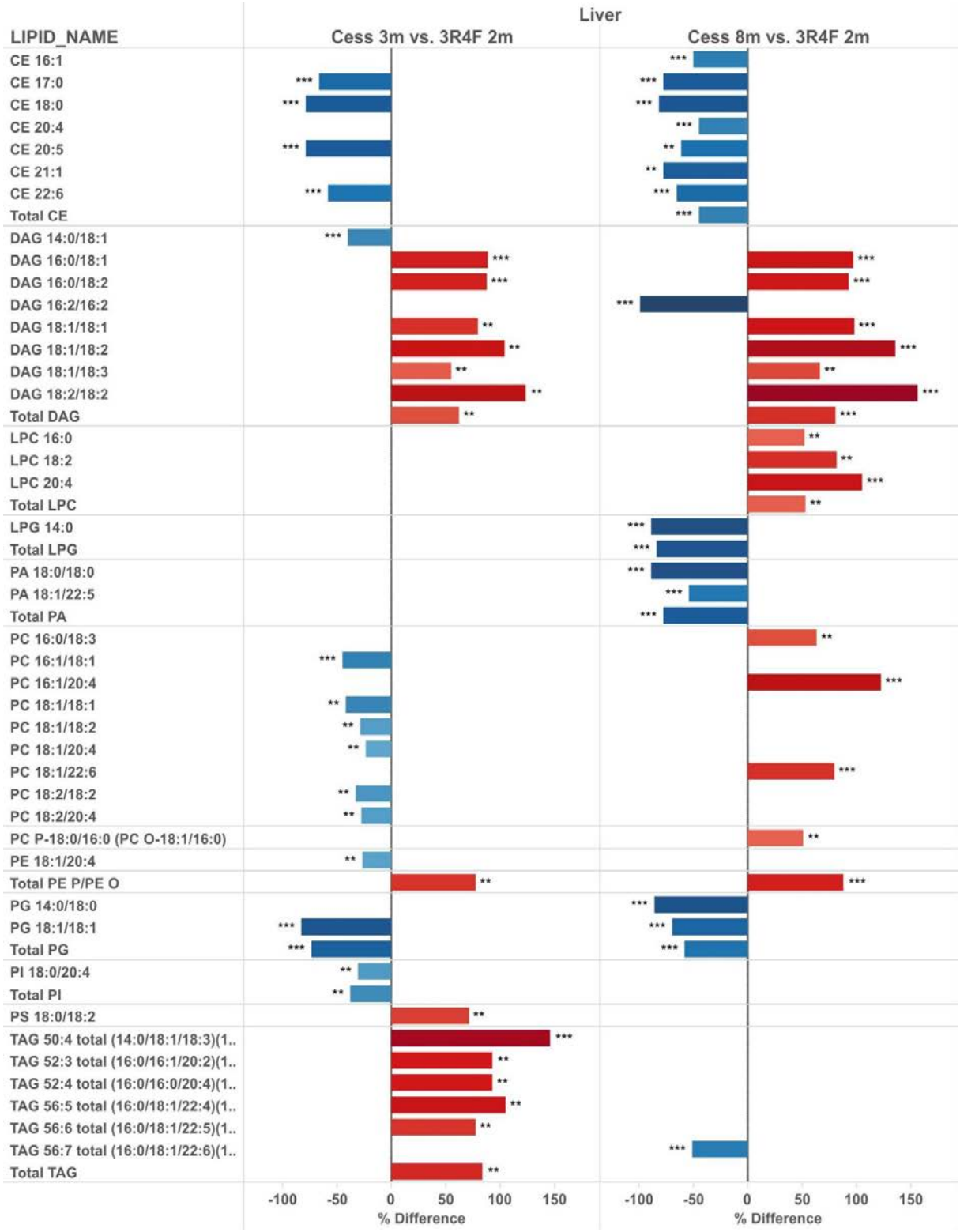



**Figure 38.** Relative percentage differences in liver in the cessation group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.5.3 Abdominal aorta

**Platforms: SG, CER, FC**

When the cessation time points (3 and 8 months) were compared to the 3R4F group at 2 month, the ceramides were increased at both time points and the cerebrosides were decreased at 8 month time point (**Figure 39**). Diacylglycerols were increased at 3 month time point, whereas phospholipids showed decrease at 8 month time point (**Figure 55**).

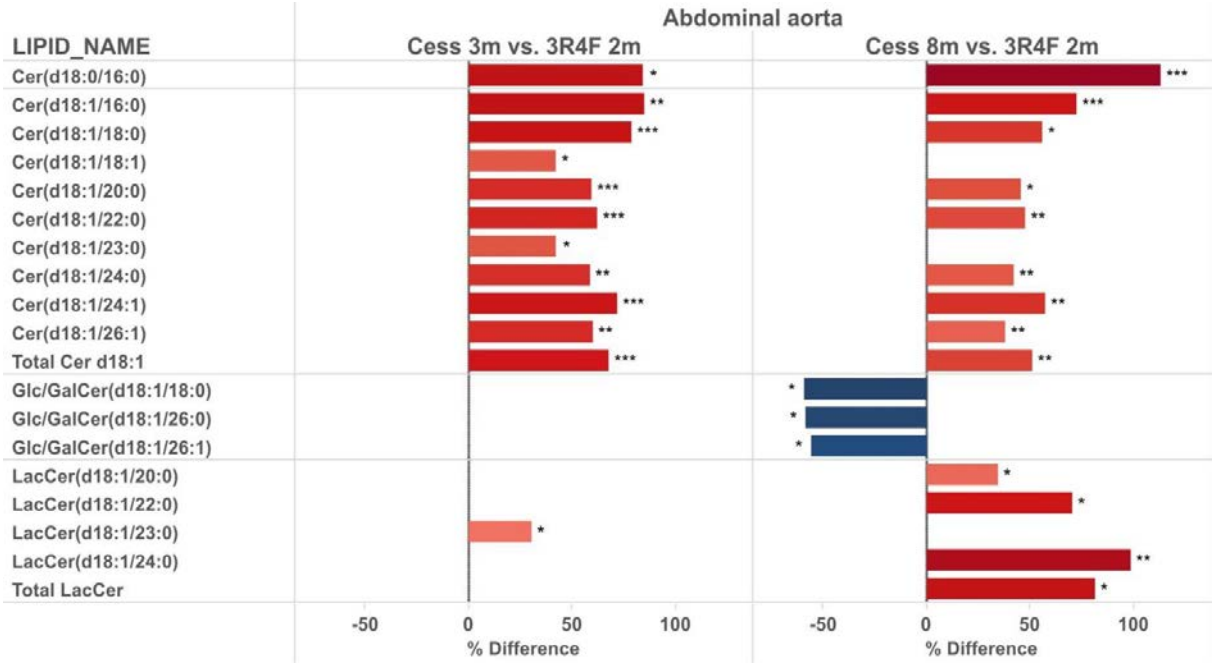



**Figure 39.** Relative percentage differences of ceramides in abdominal aorta in the cessation group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

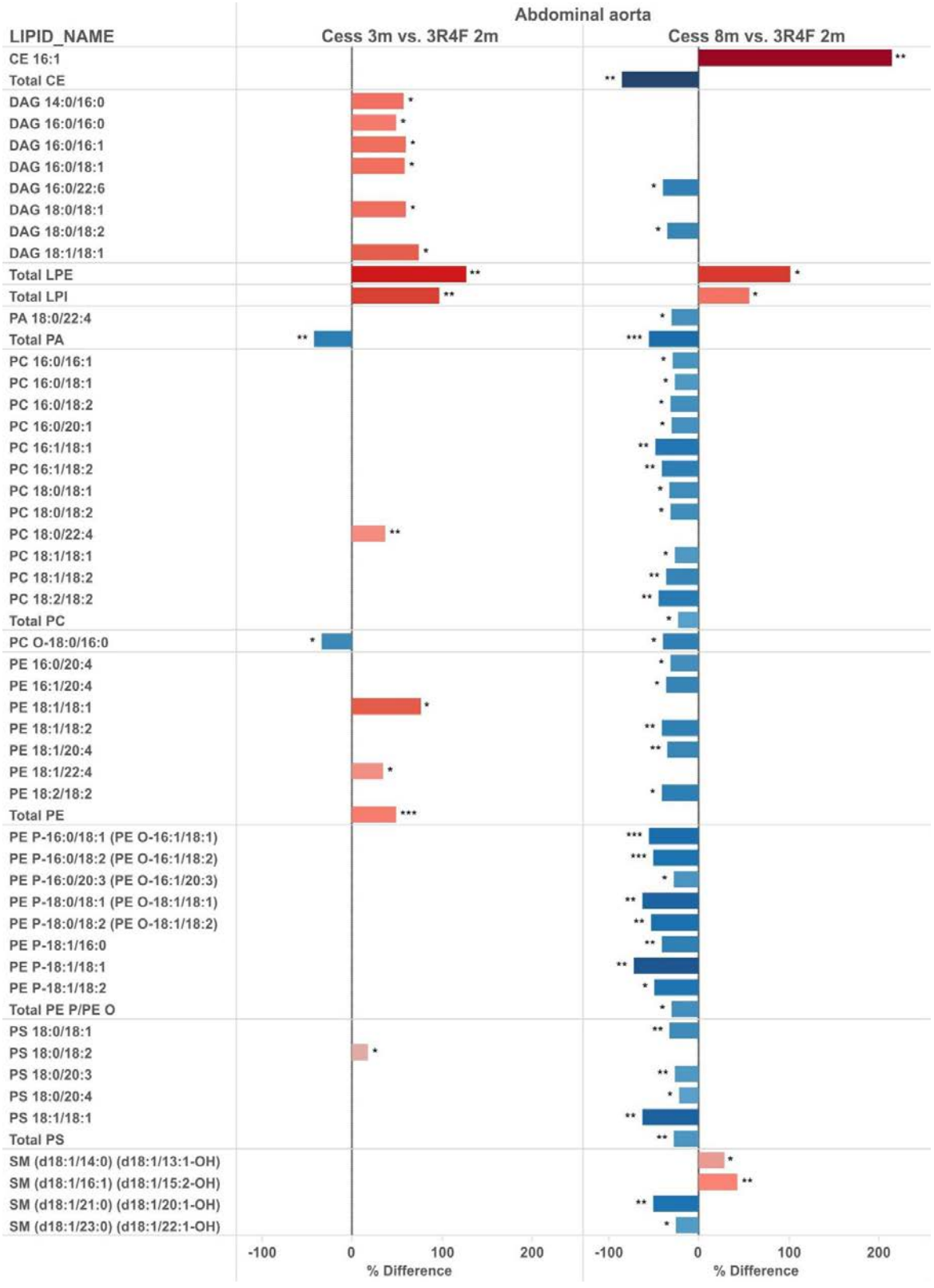



**Figure 40.** Relative percentage differences in abdominal aorta in the cessation group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.5.4 Summary

Cessation caused dramatic lipidomic changes in lung, where the phospholipids and ceramides were decreased. Ceramides were also decreased in liver, but increased in abdominal aorta. In abdominal aorta phospholipids were decreased at 8 month time point.

## 10.6 Pairwise group comparisons: switch against 3R4F at 2 months

### 10.6.1 Lung

**Platforms: SG, CER**

When the switch time points (3 and 8 months) were compared to the 3R4F group at 2 months, there was a large decrease of several lipid classes in the lung, and the results resembled those of the cessation group. Ceramides and cerebrosides, diacylglycerols, sphingomyelins **(Figure 41)**, phospholipids **(Figure 42)** and especially phosphatidylglycerols **(Figure 43)** were all decreased, and in general, the decreasing trend at 8 month time point was stronger than at 3 month time point.

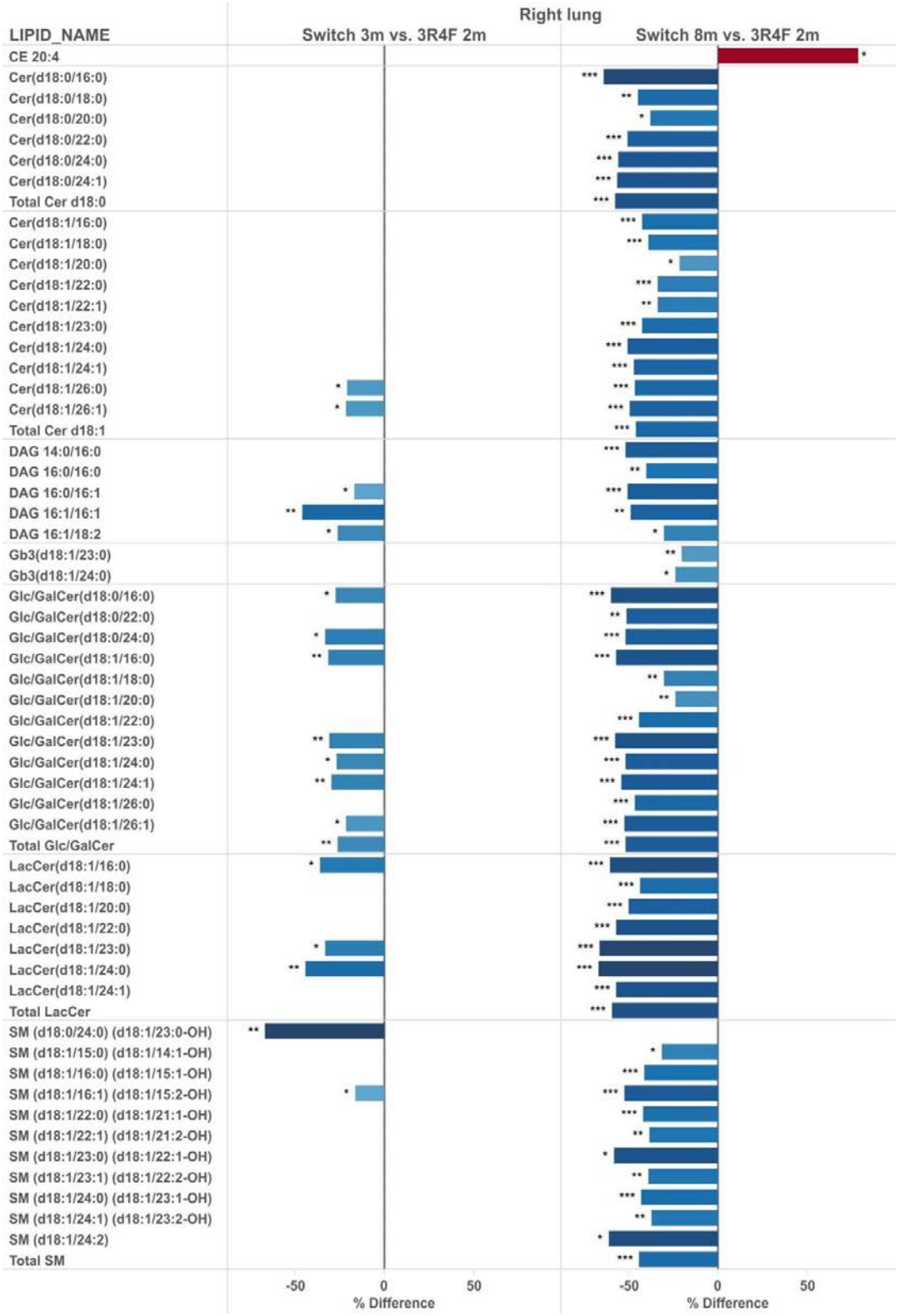



**Figure 41.** Relative percentage differences of sphingolipids, diacylglycerols and cholesteryl esters in lung in the switch group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

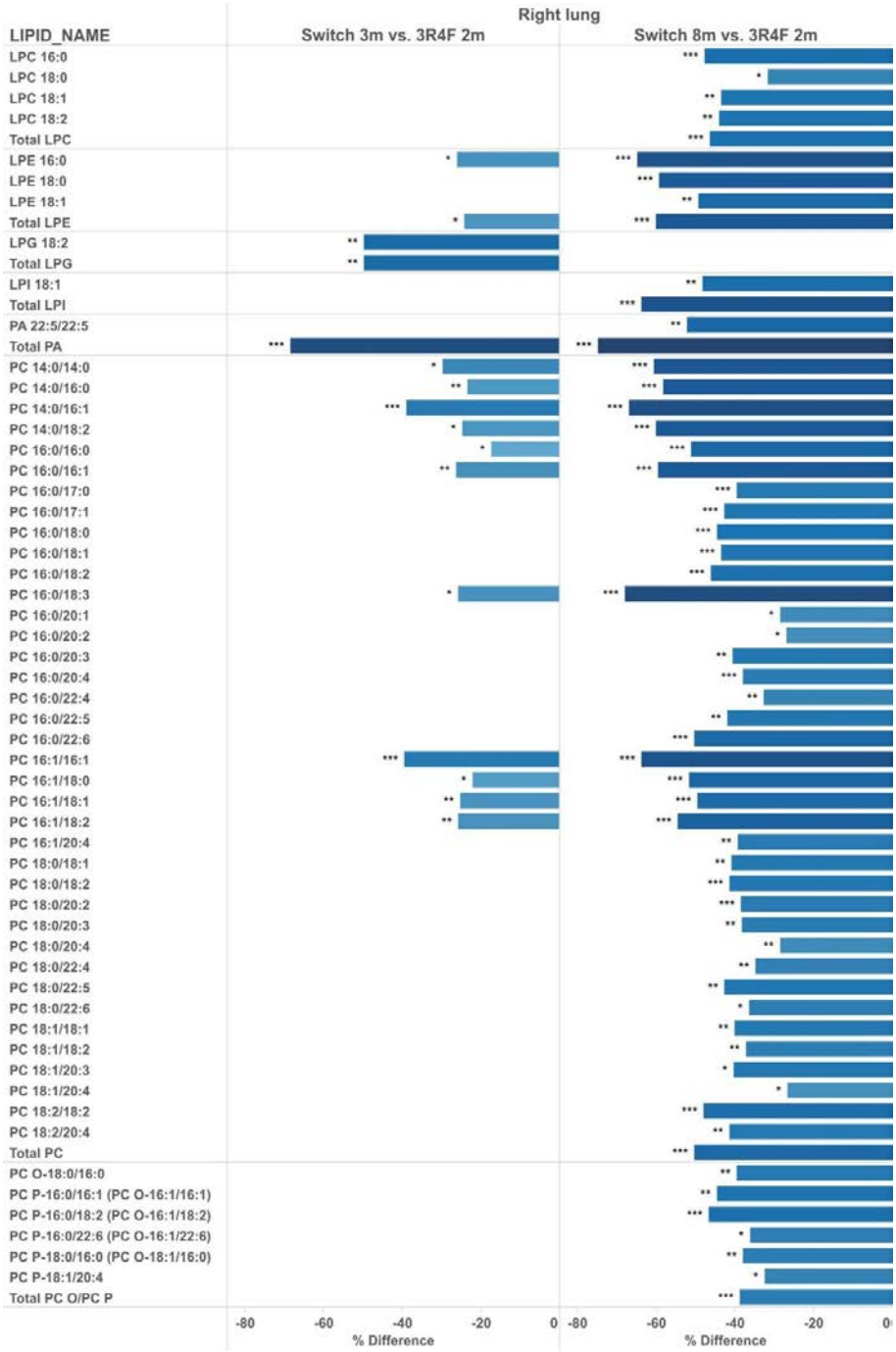



Figure continues on next page

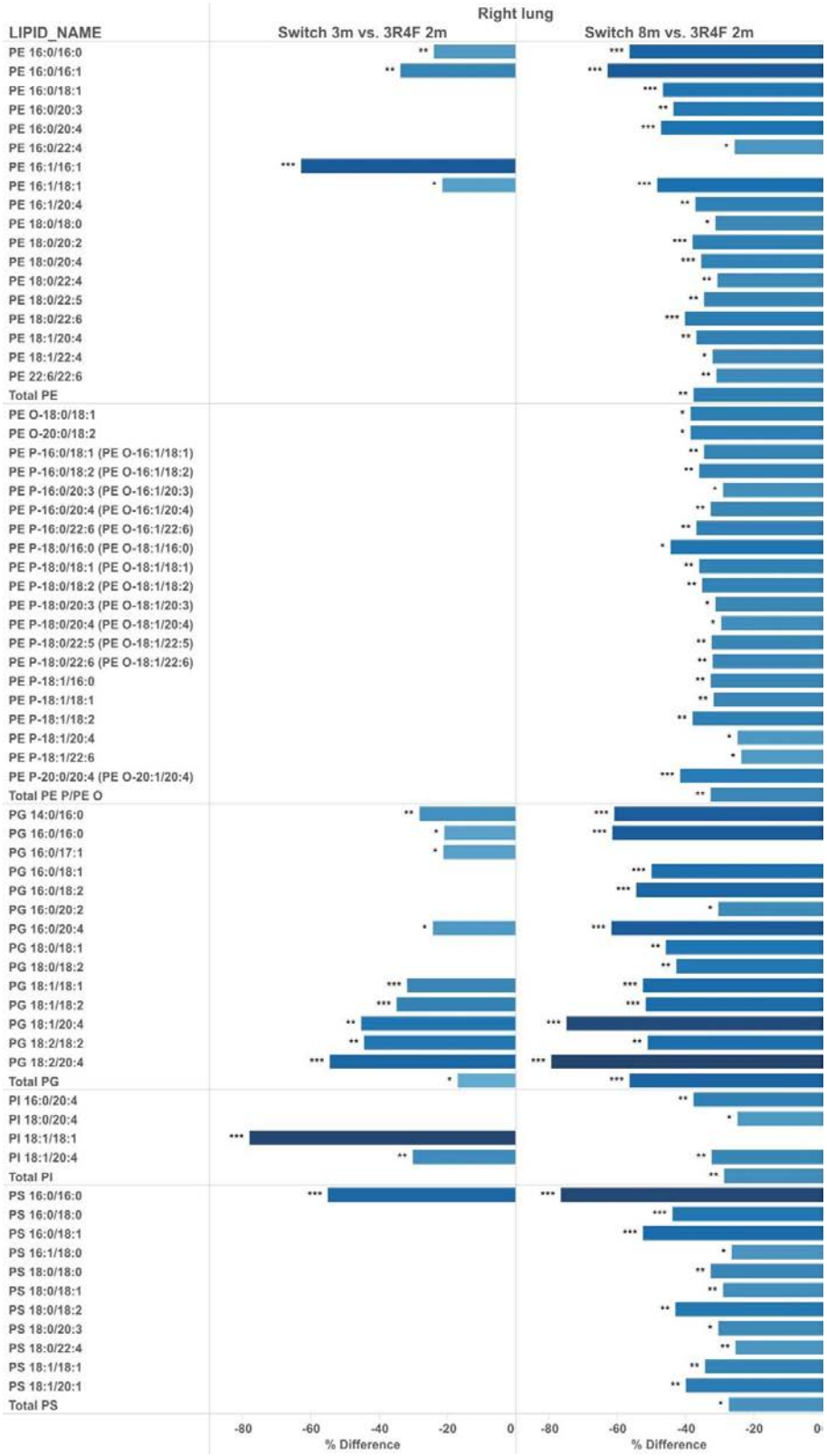



**Figure 42.** Relative percentage differences of phospholipids in lung in the switch group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

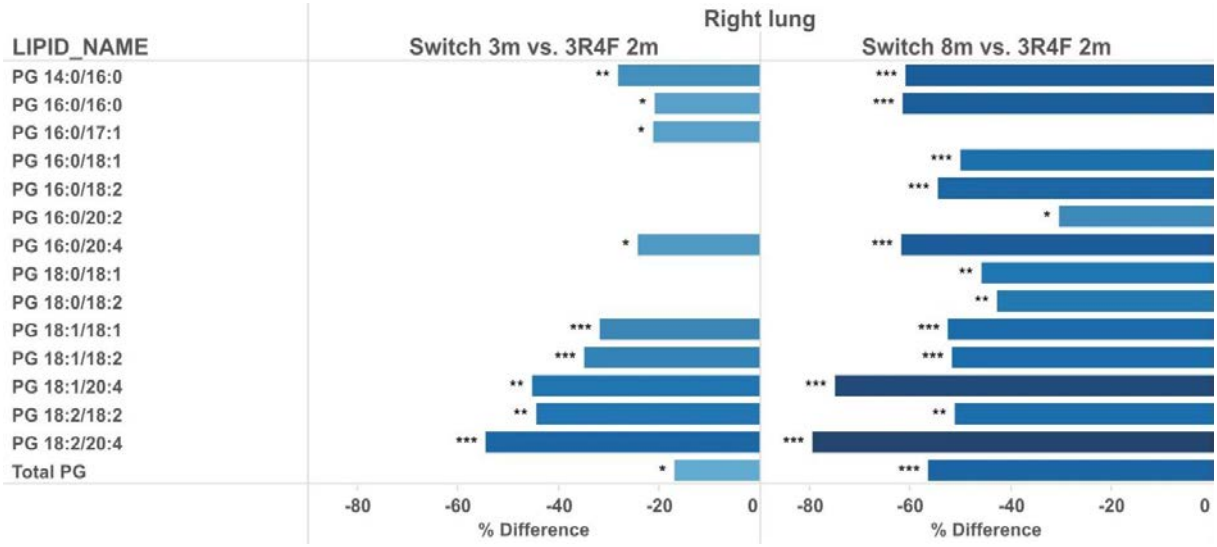



**Figure 43.** Relative percentage differences of phosphatidylglycerols in lung in the switch group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.6.2 Liver

**Platforms: SG, TAG, CER**

When the switch time points (3 and 8 months) were compared to the 3R4F group at 2 months, the results resembled those of the cessation group, as most of the ceramides were decreased at 3 month time point (**Figure 44**). This was apparent also for the d18:0-ceramides at 8 month time point, but the cerebrosides and globotriasoylceramides were increased. For the other lipids (**Figure 45**) the results of diacylglycerols resembled those of the cessation group in that they were increased at both time points.

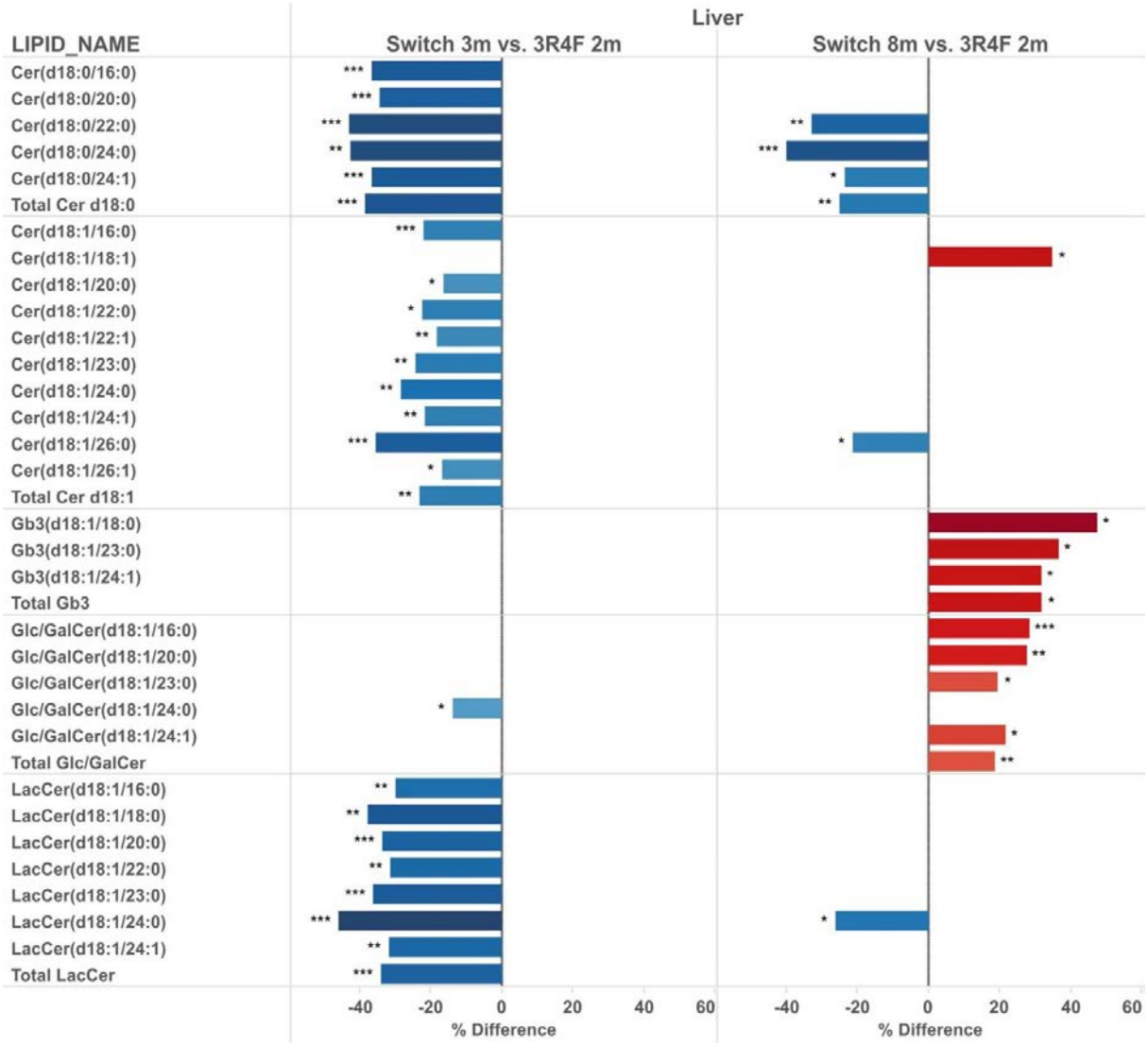



**Figure 44.** Relative percentage differences of ceramides in liver in the switch group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

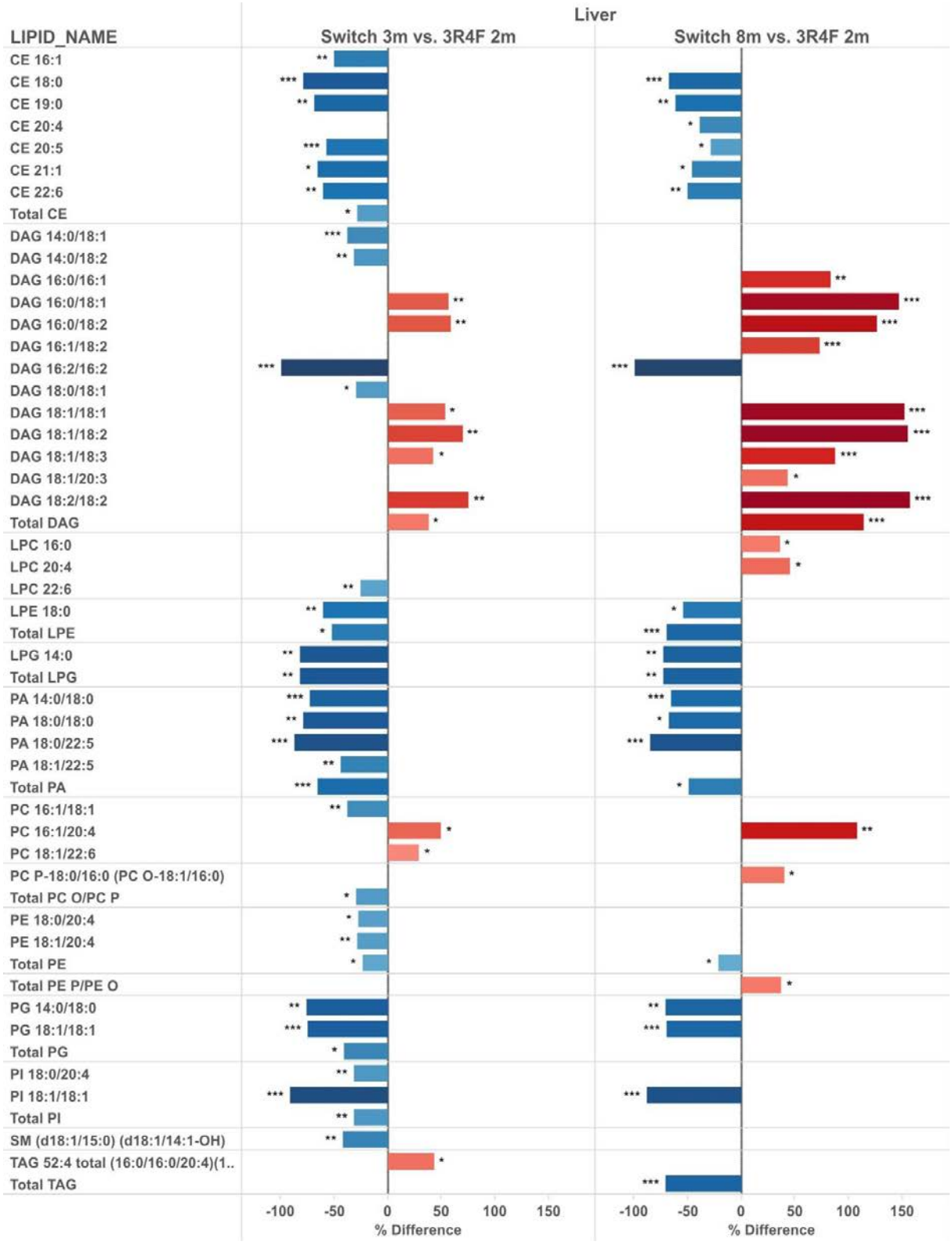



**Figure 45.** Relative percentage differences in liver in the switch group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.6.3 Abdominal aorta

**Platforms: SG, CER, FC**

When the switch time points (3 and 8 months) were compared to the 3R4F group at 2 months, the results were similar to those of the cessation group in that the ceramides were increased at both time points, diacylglycerols were increased at 3 months and phospholipids decreased at 8 months **(Figure 46)**.

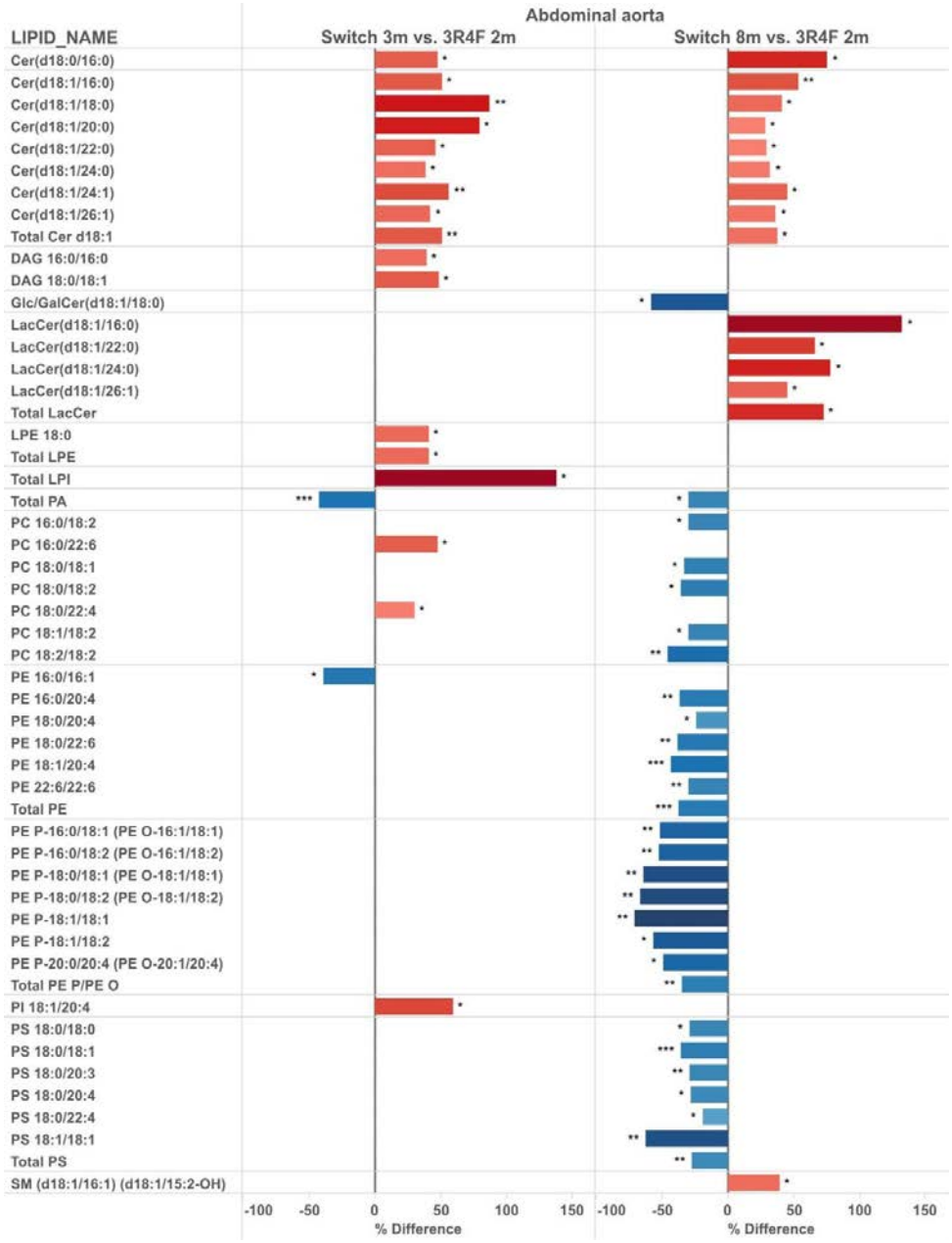



**Figure 46.** Relative percentage differences of ceramides in abdominal aorta in the switch group when compared to 3R4F 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.6.4 Summary

Switch caused similar changes as cessation in all the investigated tissues. Dramatic lipidomic changes were observed in lung, where the phospholipids and ceramides were decreased. Ceramides were also decreased in liver, but increased in abdominal aorta. In addition, cholesteryl esters were decreased and diacylglycerols increased in liver.

## 10.7 Pairwise time point comparisons: 3 and 8 vs. 2 months

### 10.7.1 Lung

**Platforms: SG, CER**

The pairwise comparisons of 3 and 8 month vs. 2 month time point are shown in **Figure 47**, **Figure 48** and **Figure 49** (only $p<0.01$ shown in the figures). Surprisingly, there were differences in the Sham group, as cholesteryl esters were increased and cerebrosides decreased at both 3 and 8 month time points. Ceramides, sphingomyelins and phospholipids were decreased at 8 month time point. For the 3R4F group, the most evident difference was the increase of phosphatidylglycerols at 8 months. The P1 group showed decreased levels of diacylglycerols, cerebrosides and lysophosphatidylcholines at both 3 and 8 months. In addition, many ether-linked phosphatidylethanolamines and phosphatidylglycerols were decreased at 8 month time point.

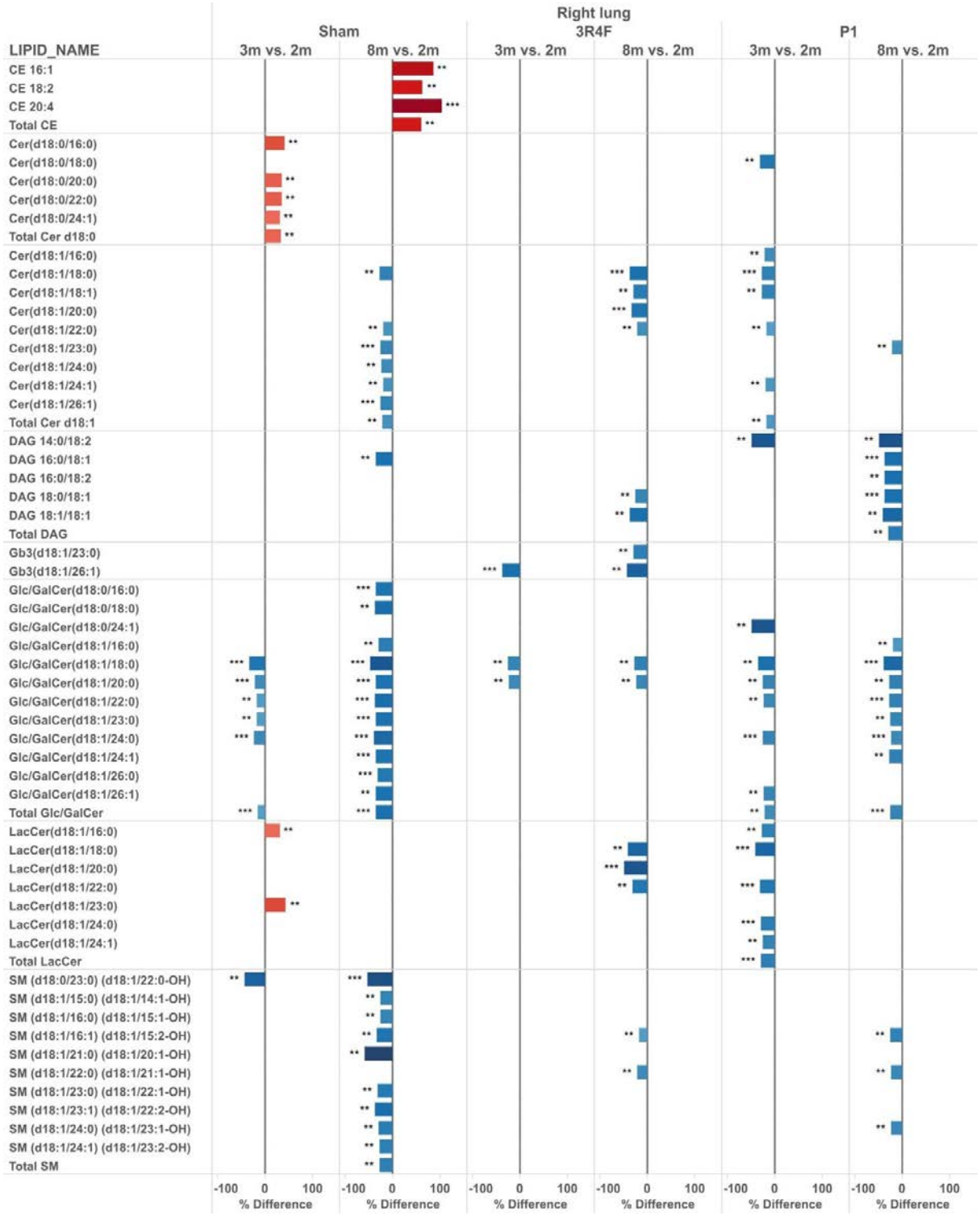



**Figure 47.** Relative percentage differences of ceramides, diacylglycerols and cholesteryl esters in lung when 3 or 8 month time point is compared to 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

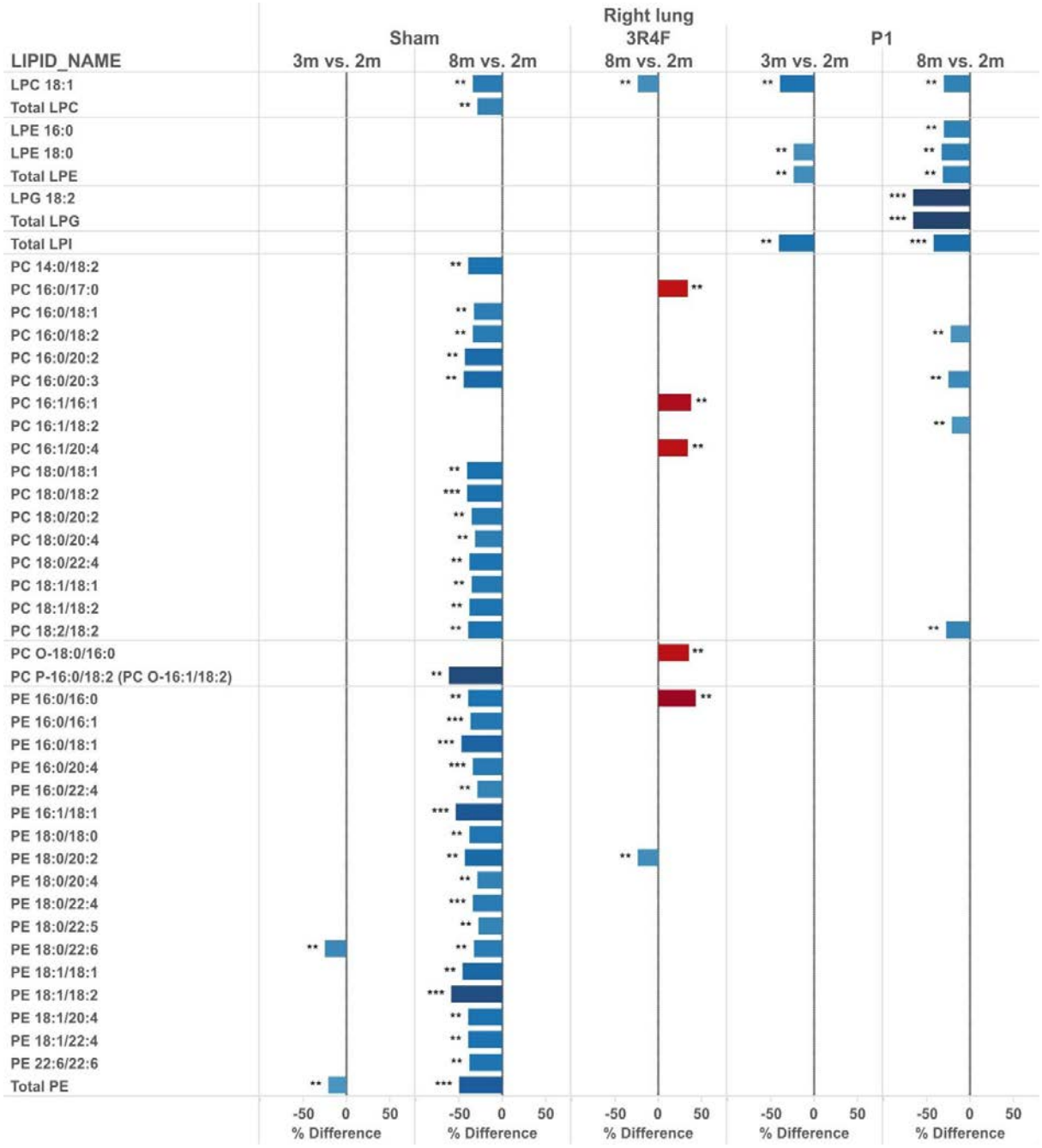



Figure continues on next page

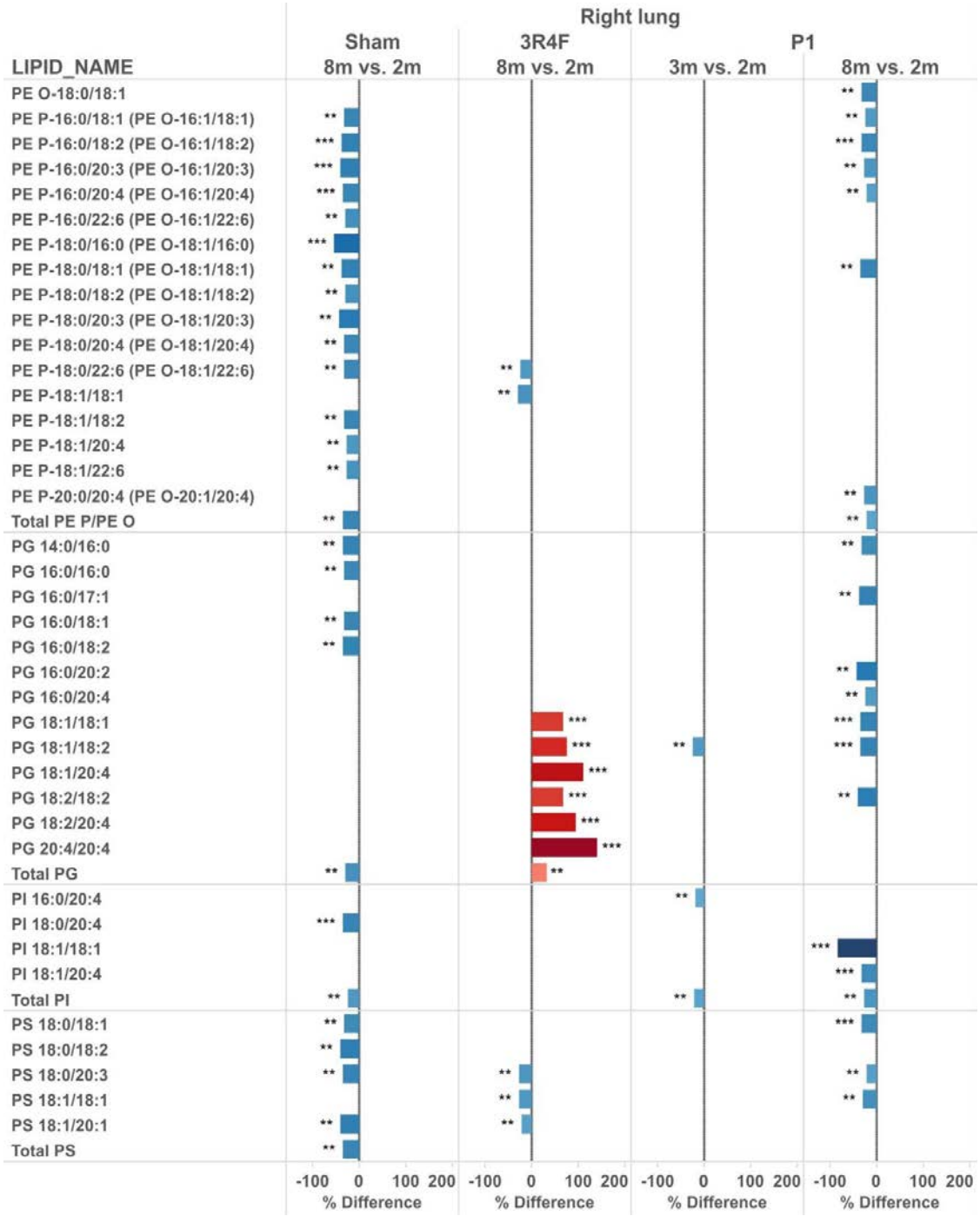



**Figure 48.** Relative percentage differences of phospholipids in lung when 3 or 8 month time point is compared to 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

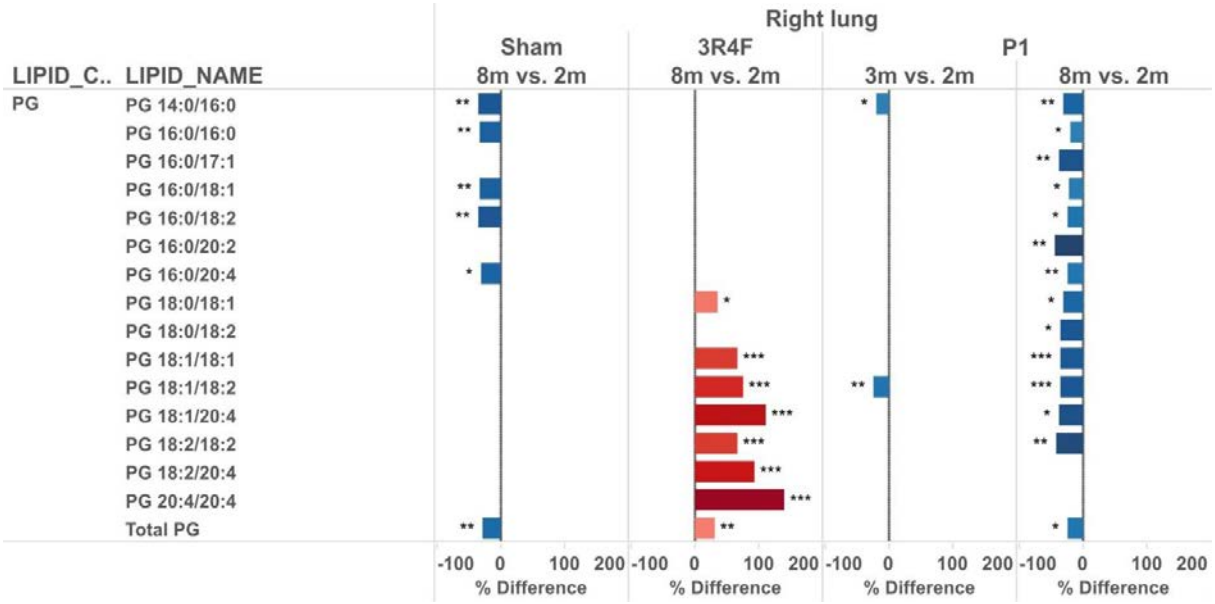



**Figure 49.** Relative percentage differences of phosphatidylglycerols in lung when 3 or 8 month time point is compared to 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.7.2 Liver

**Platforms: SG, TAG, CER**

The pairwise comparisons of 3 and 8 month vs. 2 month time point for ceramides are shown in **Figure 50** and for other lipids in **Figure 51**. Also in liver there were differences in the Sham group, as the ceramides and cerebrosides as well as diacylglycerols were increased at 8 month time point. In a similar manner the cerebrosides were also increased in the 3R4F group at 8 month time point, whereas for the P1 group at that time point there was an increase of globotriasoylceramides and decrease of lactosylceramides.

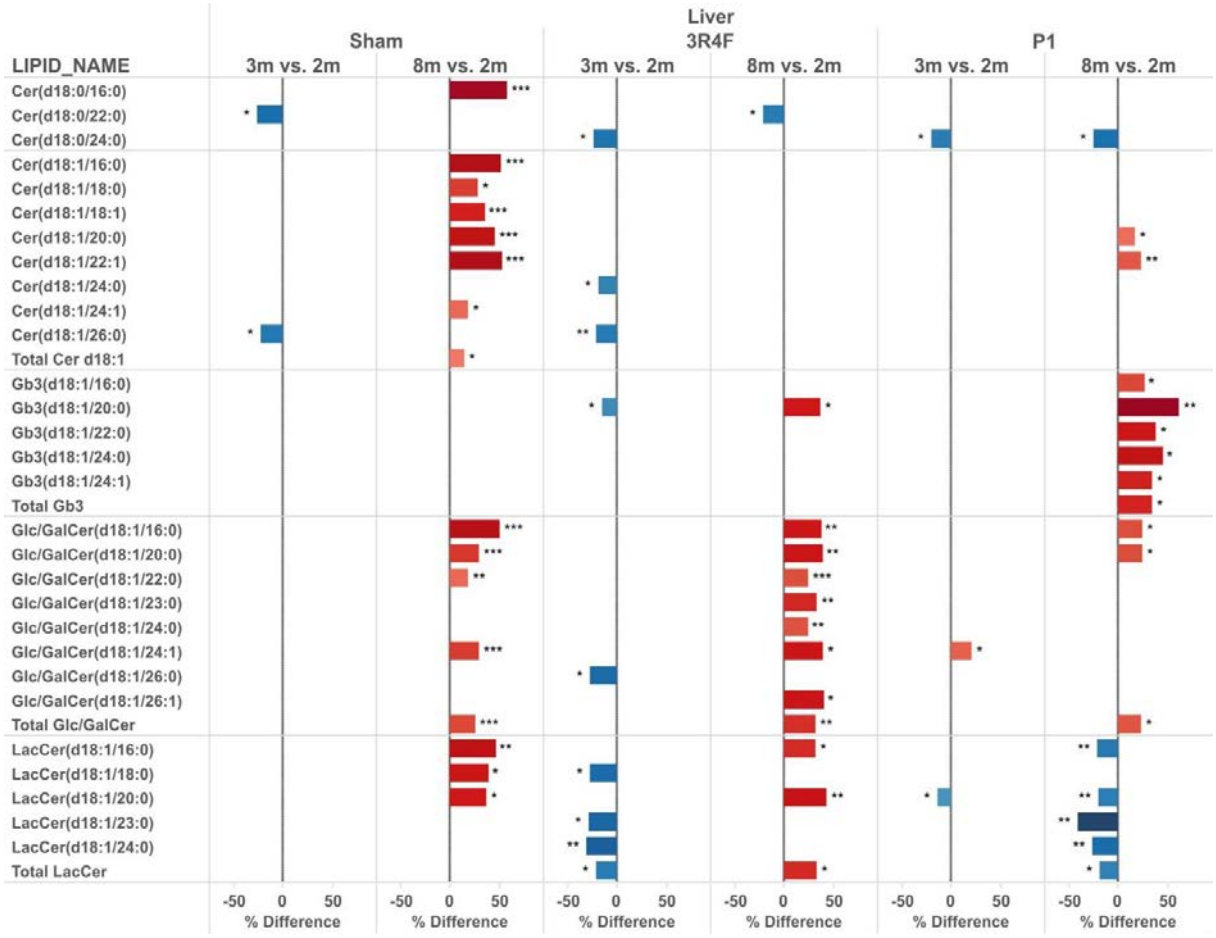



**Figure 50.** Relative percentage difference of ceramides in liver when 3 or 8 month time point is compared to 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

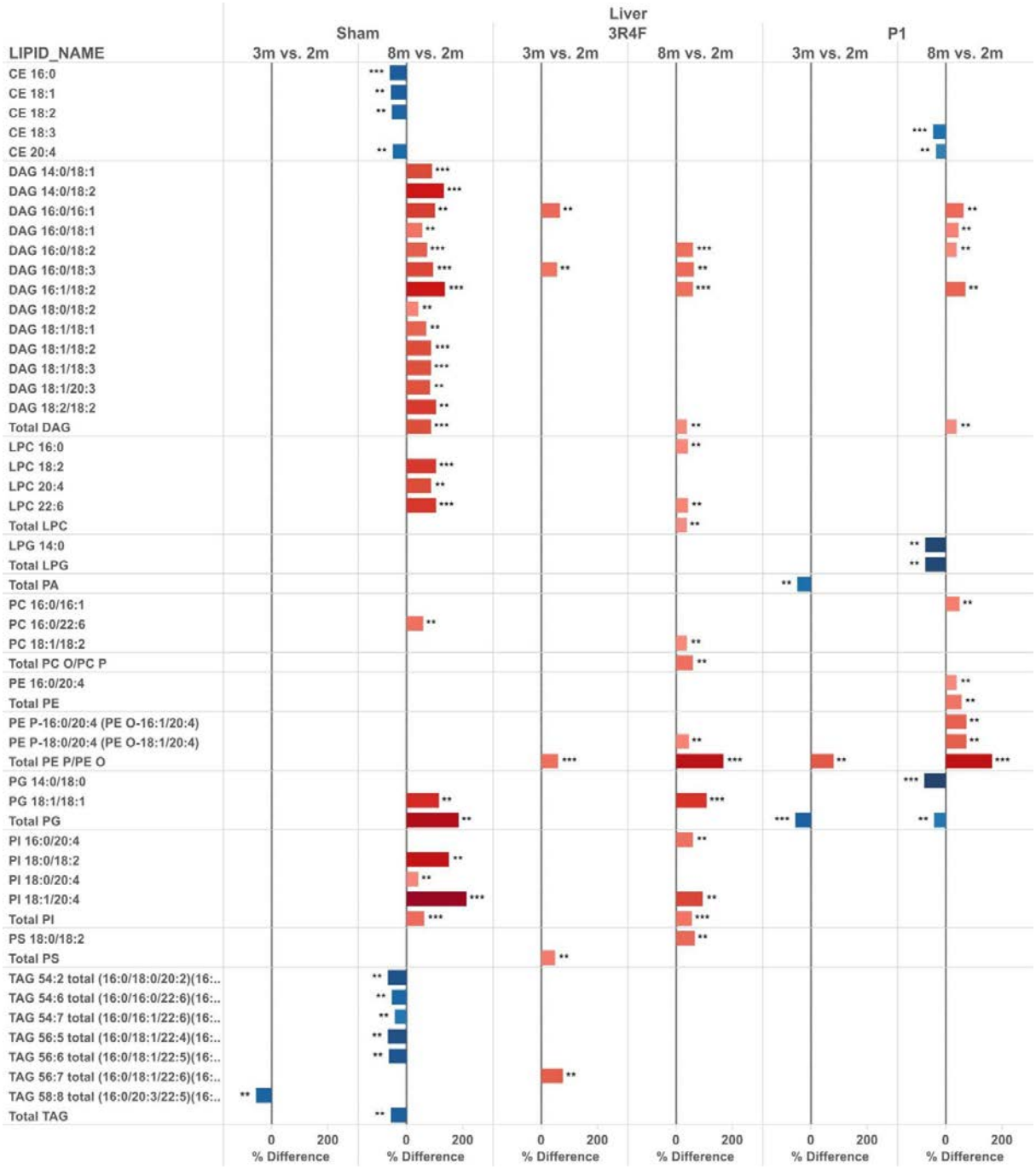



**Figure 51.** Relative percentage differences in liver when 3 or 8 month time point is compared to 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.7.3 Abdominal aorta

**Platforms: SG, CER, FC**

The pairwise comparisons of 3 and 8 month vs. 2 month time points are shown in **Figure 52** for ceramides and in **Figure 53** for other lipids. The Sham group showed decrease in glucosyl/galactosylceramides as well as phospholipids at 8 months, and for the P1 group increase in occasional lactosylceramides was observed. In the 3R4F group there was increase of lactosylceramides and cholesteryl esters at 8 month time point.

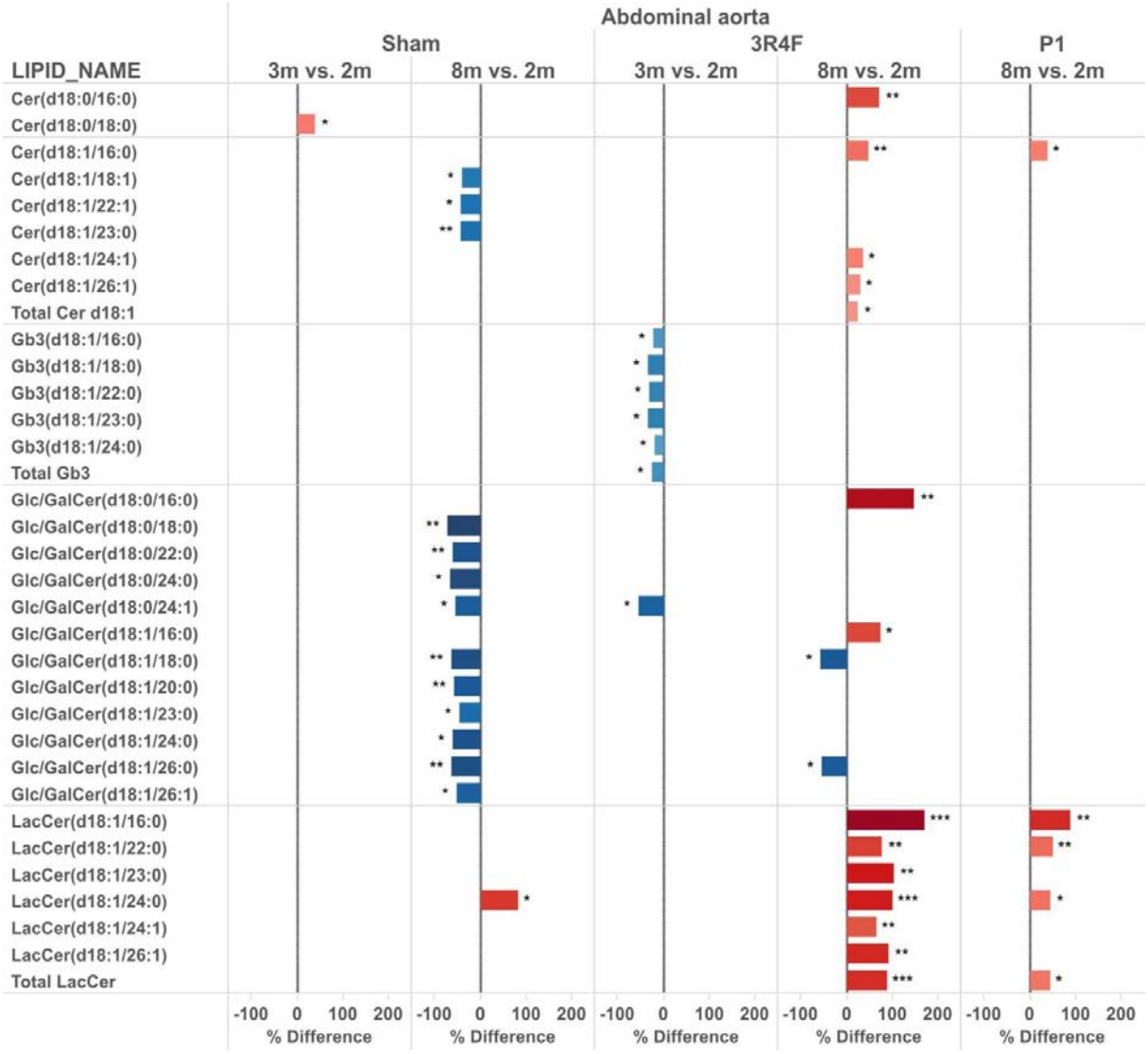



**Figure 52.** Relative percentage differences in abdominal aorta when 3 or 8 month time point is compared to 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

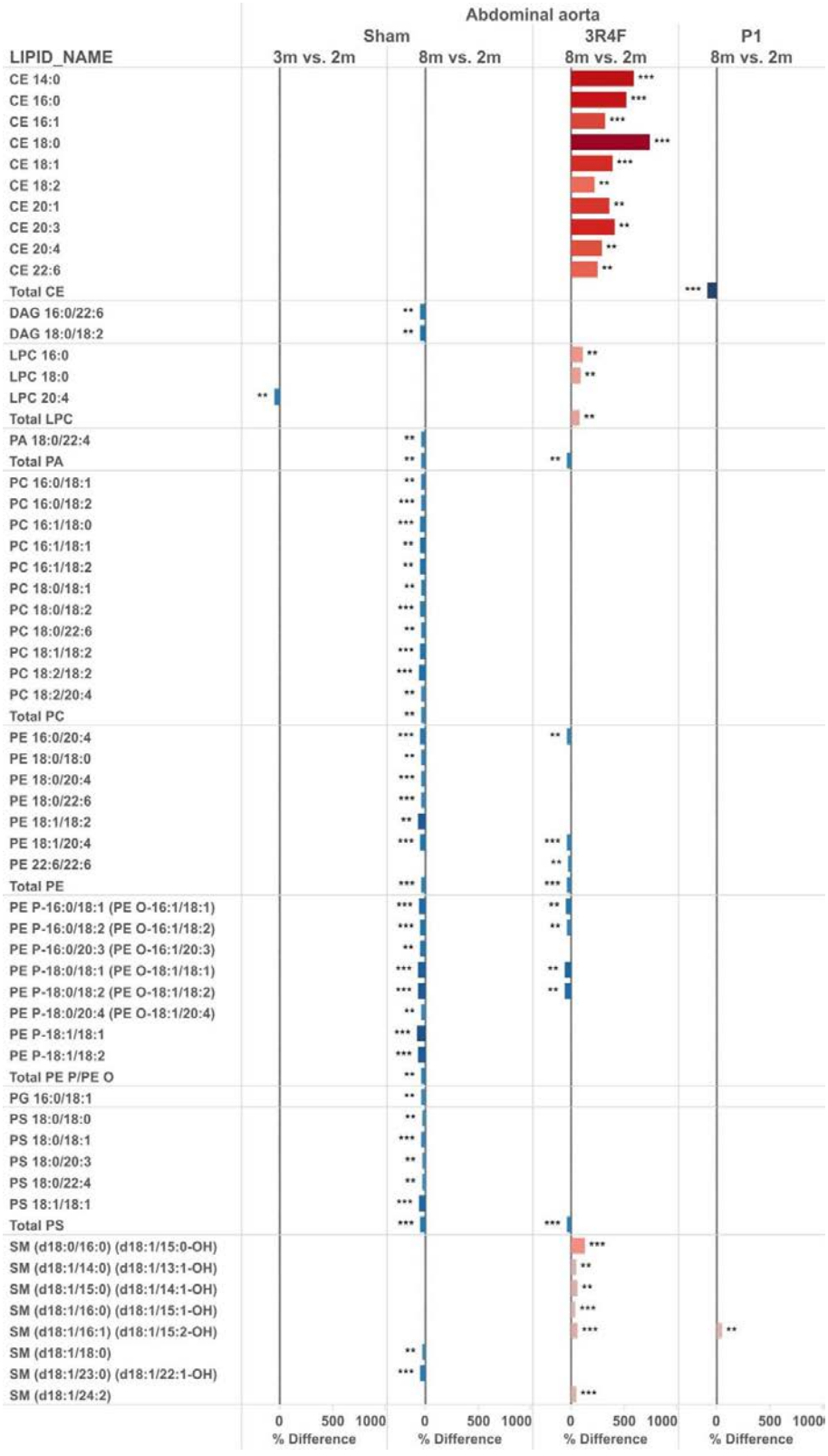



**Figure 53.** Relative percentage differences in abdominal aorta when 3 or 8 month time point is compared to 2 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.7.4 Summary

In summary, all the tissues showed changes in the Sham group, and it is possible that the genetic background of the mice may cause progressive changes. The main differences between the groups were the upregulation of phosphatidylglycerols in lung and cholesteryl esters in the abdominal aorta of the 3R4F group. Otherwise, the results were modest and suggest that most of the changes may not have been progressive and the most obvious alterations had already occurred at 2 month time point.

## 10.8 Pairwise time point comparisons: 8 vs. 3 months

### 10.8.1 Lung

**Platforms: SG, CER**

When comparing the lung lipidomic profiles at 8 month time point as compared to 3 months, the strongest changes were observed in the switch group. The results for phospholipids are shown in **Figure 54** and for the other lipids in **Figure 55**. Due to high number of changing lipids the results for phospholipids are shown only for those with $p<0.001$ and for the other lipids with $p<0.01$. The least differences were observed in the P1 group showing very stable lipid concentrations between 3 and 8 month time points. In Sham, 3R4F and cessation groups there was an overall trend of decreasing phospholipids with p-value<0.05 (please see the attached Tableau file for more details). The switch group showed the most dramatic decrease of phospholipids.

Regarding other lipids there was strong decrease of ceramides observed in several groups. The most striking difference was seen in the switch group, but also Sham group showed a decrease. 3R4F also showed a decrease, while the cessation group showed the least differences. As in other analyses, also in this case the increase of phosphatidylglycerols was evident in the 3R4F group.

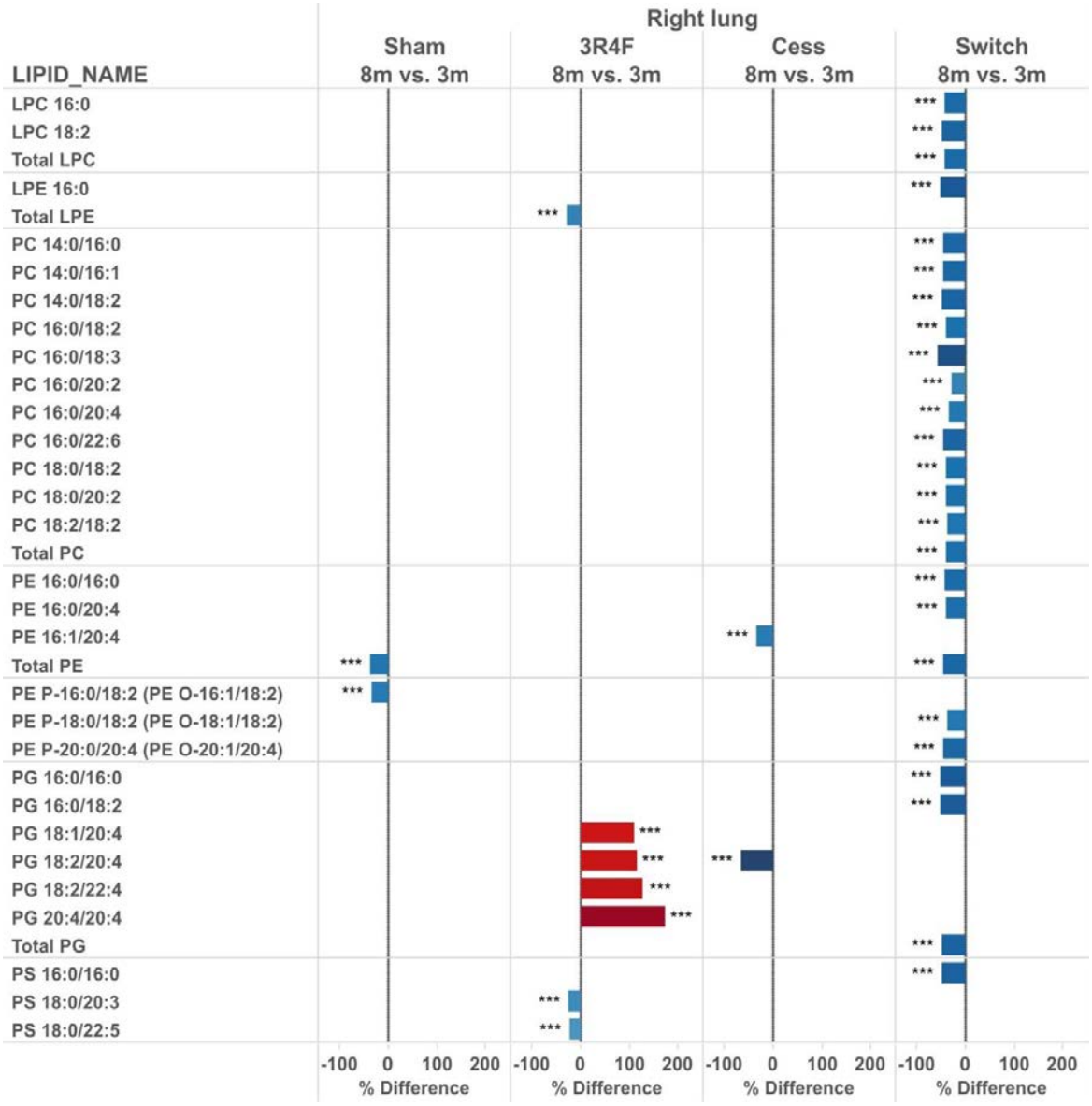



**Figure 54.** Relative percentage differences in lung when 8 month time point is compared to 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

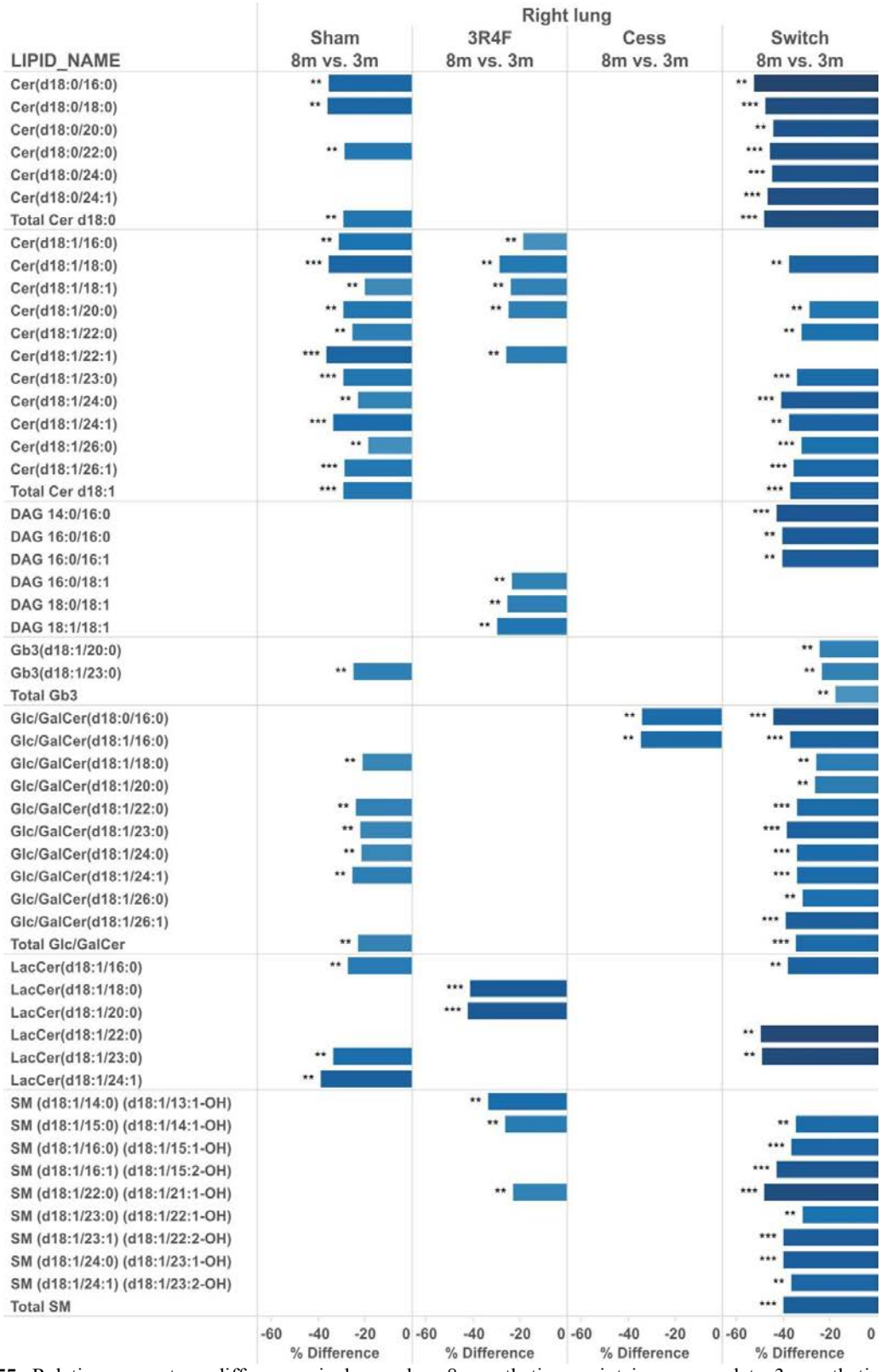



**Figure 55.** Relative percentage differences in lung when 8 month time point is compared to 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.8.2 Liver

**Platforms: SG, TAG, CER**

The comparison of liver ceramides between 8 month and 3 month time point is shown in **Figure 56** and for other lipids in **Figure 57**. P1 group did not show major differences, but the increase of ceramides was evident in all other groups. The strongest changes were the increase of lactosylceramides in the 3R4F group and increase of globotriasoylceramides in the cessation group. In the sham group diacylglycerols were increased and triacylglycerols decreased and in the cessation group cholesteryl esters were decreased and phosphatidylcholines increased.

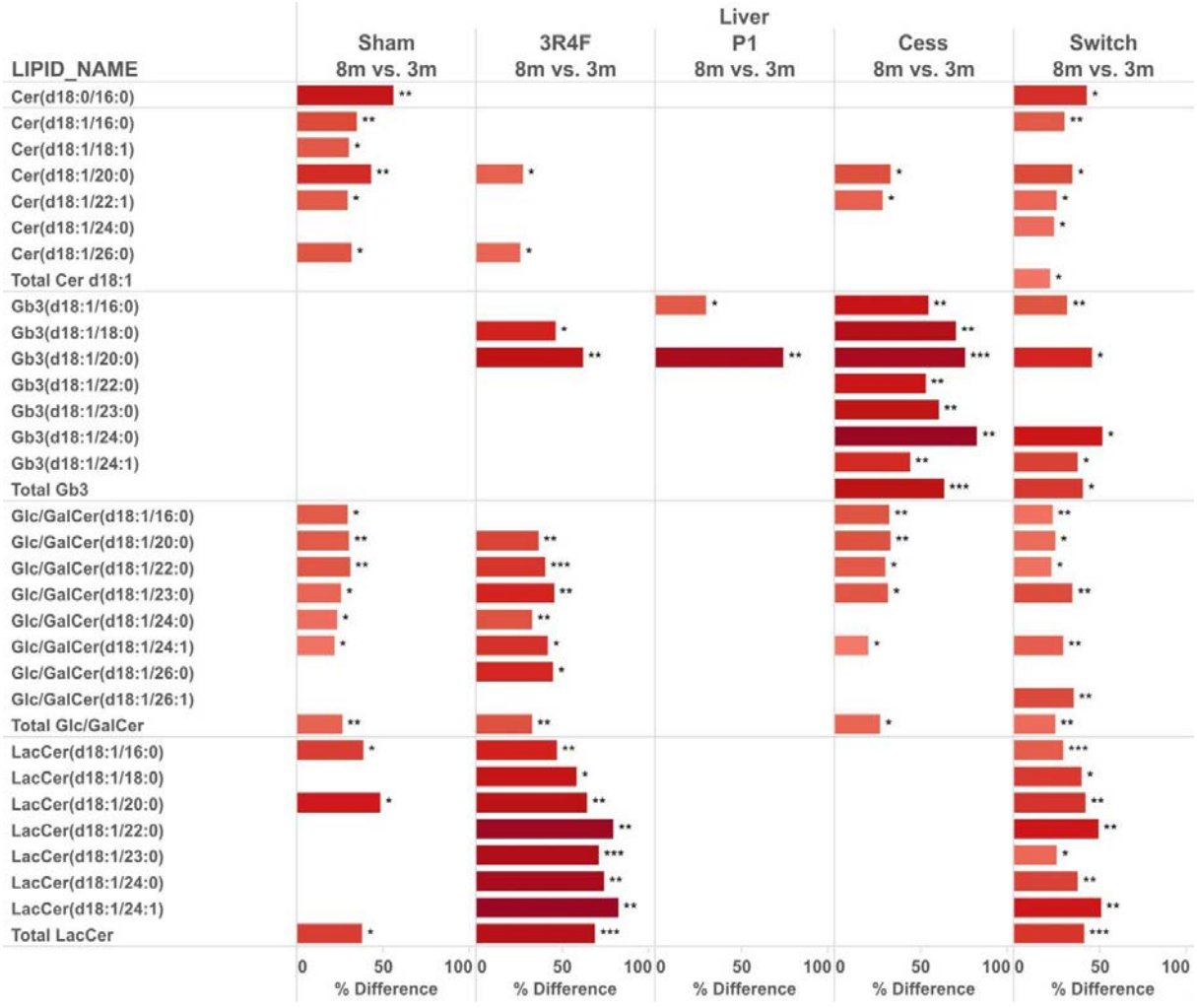



**Figure 56.** Relative percentage differences of ceramides in liver when 8 month time point is compared to 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

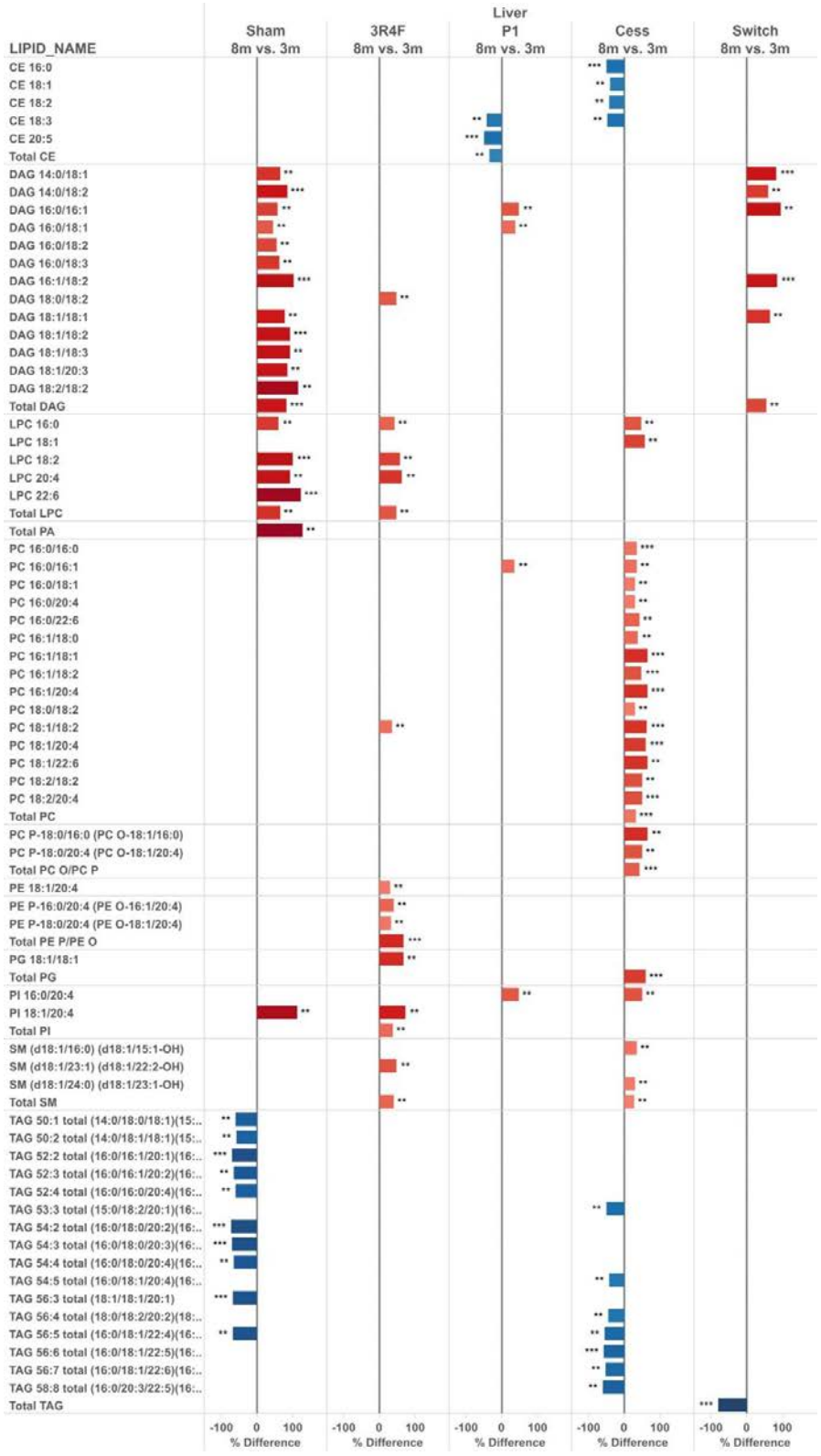



**Figure 57.** Relative percentage differences in liver when 8 month time point is compared to 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.8.3 Abdominal aorta

**Platforms: SG, CER, FC**

The results for abdominal aorta comparison between 8 and 3 month time points are shown in **Figure 58** for ceramides, in **Figure 59** for cholesteryl esters and in **Figure 60** for other lipids. In ceramides, the most obvious changes were the increase of lactosylceramides in the Sham and 3R4F groups. Cholesteryl esters showed high increase in the order of 500-1000% in the 3R4F group. In the 3R4F group also lysophosphatidylcholines and sphingomyelins were increased and diacylglycerols decreased. Diacylglycerols were also decreased in the cessation group, and phospholipids showed decrease in the sham, cessation and switch groups.

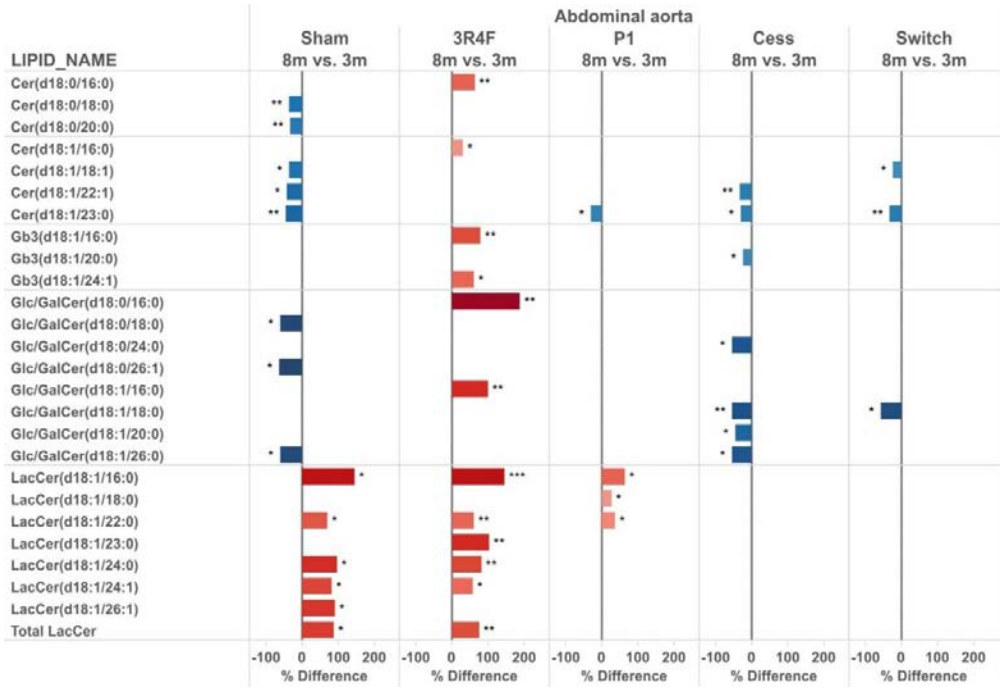



**Figure 58.** Relative percentage differences of ceramides in liver when 8 month time point is compared to 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

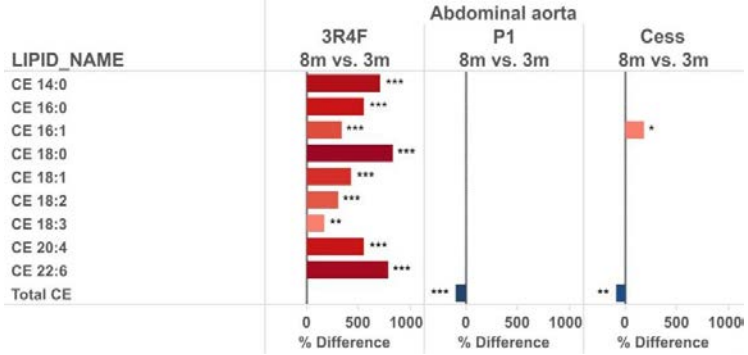



**Figure 59.** Relative percentage differences of cholesteryl esters in liver when 8 month time point is compared to 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

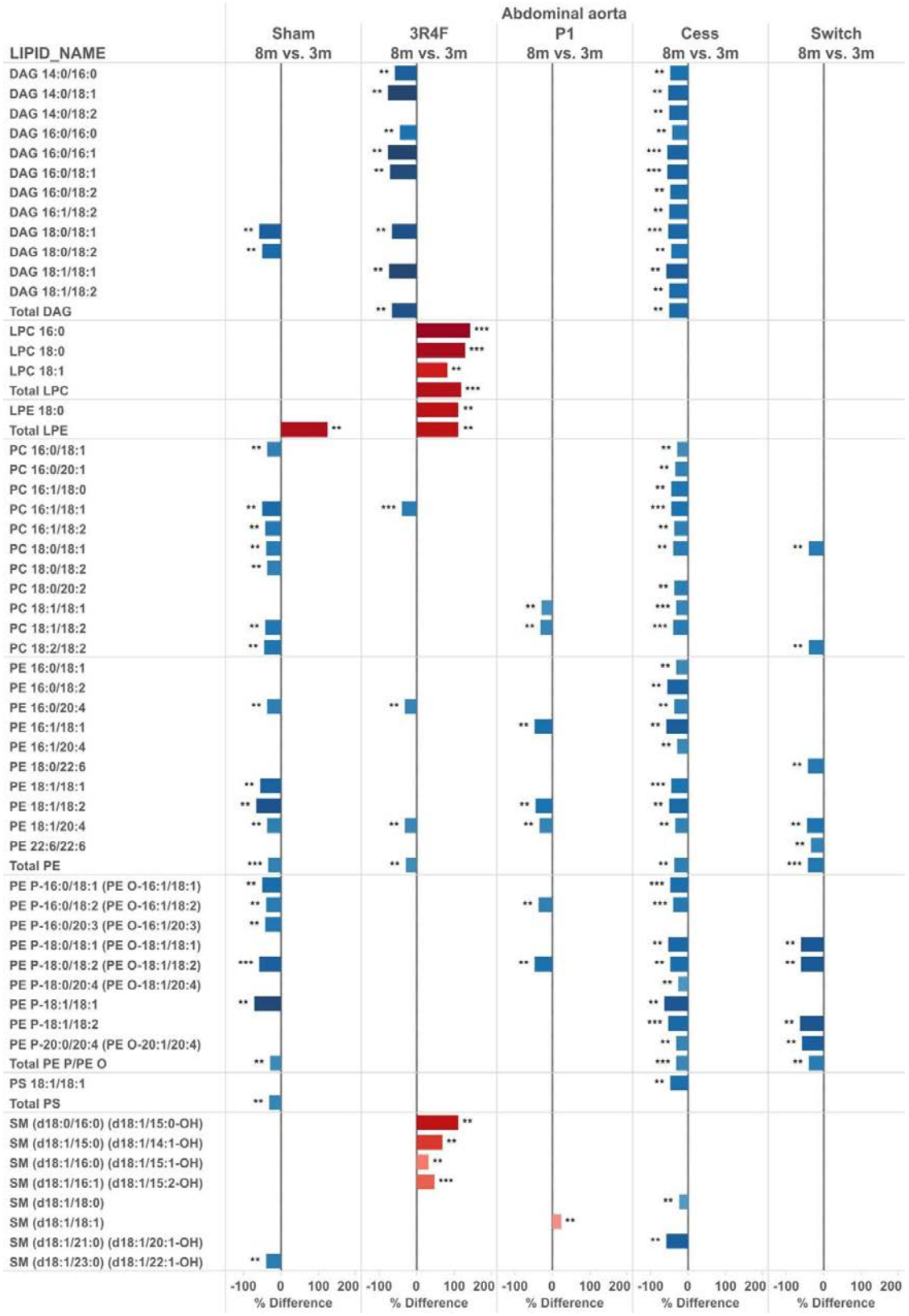



**Figure 60.** Relative percentage differences in liver when 8 month time point is compared to 3 month time point. Significance of values as *p<0.05, **p<0.01, and ***p<0.001.

### 10.8.4 Summary

In lung, the switch group showed a large decrease in lipid levels, but the results were puzzling because also the Sham group showed decrease, and in the cessation group the changes were less radical. In liver, the ceramides were increased at 8 month time point in the switch and cessation groups, but also Sham and 3R4F groups showed a similar trend. In abdominal aorta, the greatest change was the increase of cholesteryl esters in the 3R4F group, a phenomenon, which was not observed for the other groups.

# 11 SUMMARY AND DISCUSSION

## 11.1 Plasma

The plasma samples were only available from 8 month time point. The results indicated that the P1 group differed from the other groups, and the most striking difference was the upregulation of triglycerides.

When comparing the results of the present study to previous studies (51801 and the article by Boue et al., Atherosclerosis, 2012) some of the results were consistent with the previous findings, but there were also notable differences. Free cholesterol was non-significantly elevated in the 3R4F group both in the previous and the current study. The triglyceride result was clearly different, as in the previous study the triglycerides were statistically non-significantly decreased in the 3R4F group, while in the present study all the triglycerides were increased and many of the TAG species showed a statistically significant result. It is worth noting that in the present study e.g. the Sham group had 8 animals, while in the previous study only 4 animals were included in the group when TAGs had been analyzed. According to the present study the exposure to 3R4F would affect differently the triacylglycerols than the P1 exposure.

In the previous (51801) study, in general, the ceramides showed elevated levels, although e.g. the total levels did not show statistically significant increase (Fig 1. in the publication). In the present study d18:0 dihydroceramides were also slightly increased in the 3R4F group, while the other ceramide classes in general did not show increase or decrease. However, an interesting finding is that in the previous study three ceramides were decreased due to 3R4F exposure, these were Cer(d18:1/18:0), Glc/GalCer(d18:1/18:0) and LacCer (d18:1/18:0). Also in the present study these exactly same ceramides showed the greatest decrease in the 3R4F group, and for the Cer(d18:1/18:0) and LacCer(d18:1/18:0) lipids the result was statistically significant, while the Glc/GalCer(d18:1/18:0) did not reach statistical significance. In addition, in the present study Glc/GalCer(d18:0/18:0) was statistically significantly decreased in the 3R4F group. It is noteworthy that in general for these lipids the other groups (P1, switch, cessation) did not show as strong decrease as for the 3R4F group.

Also the results from a previous study in C57 mice (51803) showed decrease of Cer(d18:1/18:0) and Glc/GalCer(d18:1/18:0) by cigarette smoke suggesting that these lipids are robust biomarkers for the changes caused by cigarette smoke in plasma.

In conclusion, the free cholesterol and ceramide results were mainly consistent with the mice analyzed in the previous study at 6 month time point (in the present study 8 month time point). However, for the triglycerides the results were differing, but it is interesting that in the present study the triglyceride results were clearly separating the 3R4F group from the other groups.

## 11.2 Lung

In lung the lipidomic differences between the P1 and other groups were broad and clear. Especially phospholipids were increased in the 3R4F group, and switch to P1 or cessation resulted in decrease of the levels. The most striking difference was observed for phosphatidylglycerols that are pulmonary surfactants, and the total level of these lipids at different time points is illustrated in **Figure 61**. Also the levels of ceramides and cerebrosides were elevated in the 3R4F group.

The effect of cigarette smoke on phospholipids and ceramides has not been previously studied in ApoE KO mice, but in C57 mice in the COPD study (51803). The upregulation of phospholipids, and especially phosphatidylglycerols is consistent with the previous study. In addition, the ceramides were upregulated by cigarette smoke in both studies. Thus, it is clear that the cigarette (3R4F) smoke induces clear lipidomic changes in the lung, whereas this is not observed for the P1 exposure, and switch or cessation alleviates the changes caused by 3R4F.

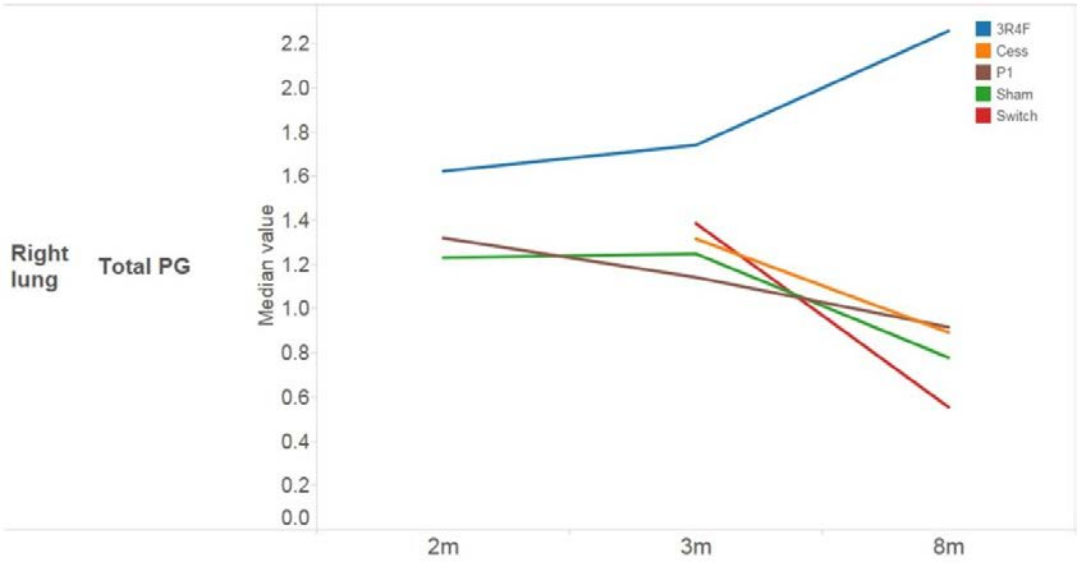



**Figure 61.** The level of total phosphatidylglycerols in different study groups.

## 11.3 Liver

The liver results were somewhat challenging to interpret. For instance when comparing to the Sham group, the ceramides displayed a decreasing trend across all time points and all groups. In addition, e.g. at 8 month time point 3R4F showed the least differences in the comparison against the Sham group and thus showing a distinct pattern compared to all the other group changes. An explanation for this phenomenon may be provided by closer inspection of the individual concentration values for each animal, in particular the Sham group.

In order to understand better the data the raw concentration values for each sample was plotted, and the plot is shown in **Figure 62**. In the plot the data for four ceramides are shown, that were representative examples of the data. From the figure it can be seen that the samples were run in two plates, and there is no major difference observed in the mean levels of groups between the plates. However, the sham groups showed higher variation, and especially the sham group at 8 month time point showed high variation and clearly elevated levels as compared to other groups. This phenomenon explains why in the results the other groups showed decreased levels of ceramides when the comparisons were made against the sham group. The different behavior of the sham 8 month group is not due to analytical reasons, as the samples in the plate were in a random order and the phenomenon is not observed for other groups. Thus, this may represent a true biological result, but it is also important to check whether the samples from mice have been taken and prepared in an identical way for each group.

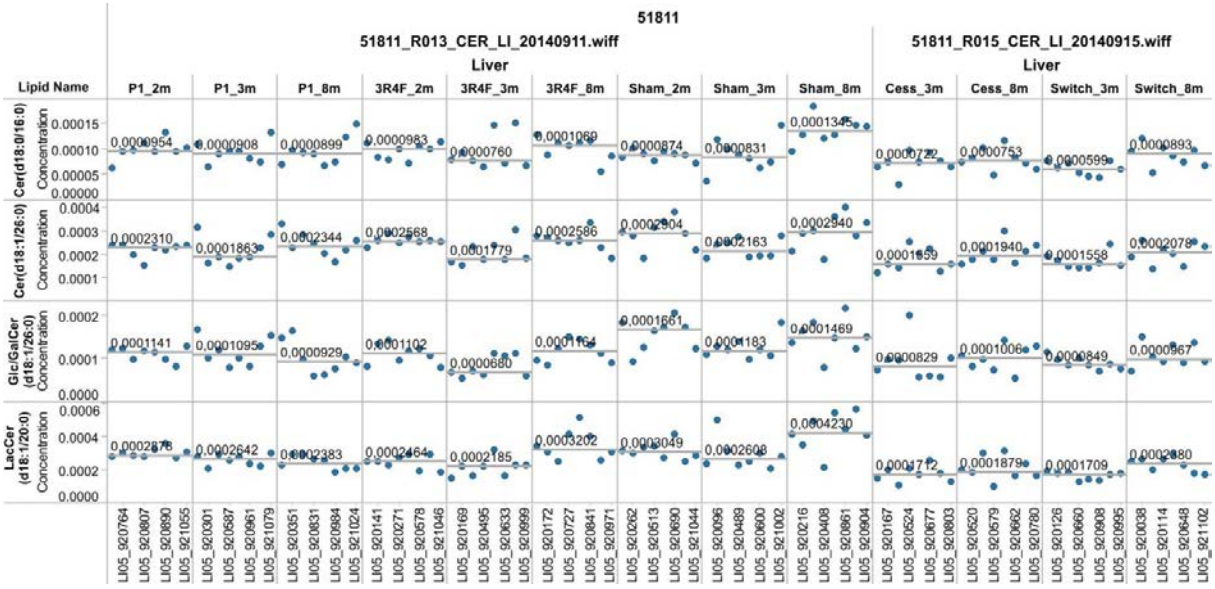



**Figure 62.** Raw concentration values for selected representative ceramides (Cer(d18:0/16:0), Cer(d18:1/26:0), Glc/GalCer(d18:1/26:0) and LacCer(d18:1/20:0)).

The previous studies (51802 and 51803) have not reported major differences in ceramides after cigarette smoke exposure. In 51802 and in the paper by Boue et al. (Atherosclerosis, 2012) there was a slight decreasing trend of ceramides, cerebrosides and globotriasoylceramides in the cigarette smoke exposed group. In contrast, in the 51803 study there was a slight increasing trend for these lipids.

Regarding the other lipids than ceramides the previous studies have reported slightly mixed results, and in many cases the changes have not reached statistical significance. In the ApoE KO mouse study (51802) TAGs were increased after cigarette exposure while C57 mouse study (51803) showed the opposite result. In the present study the results at 2 and 3 month time point showed a decrease in triacylglycerols in the 3R4F group. In addition, at these time points the 3R4F group showed increase in phosphatidylcholines, and the increase of phospholipids was showed also in the 51802 study.

## 11.4 Abdominal aorta

The data for abdominal aorta showed some unexpected results, as e.g. the switch and cessation groups showed upregulation of several lipid species at different time points. The most logical results obtained from the abdominal aorta samples was the strong upregulation of cholesteryl esters in the 3R4F group, when the 8 month time point was compared to the 3 month time point.

With the present study it seems that the results from aortic arch are consistent with the findings from the previous studies, while the abdominal aorta shows slightly puzzling results for other lipids than cholesteryl esters. One of the reasons may be related to the samples, as the abdominal aorta samples in the study showed higher variation in their weights as compared to the aortic arch. The reason for this may be that aortic arch is a more specific anatomical structure than abdominal aorta, and therefore the dissection of the samples from mice could be more accurate. However, the most crucial aspect to keep in mind is that aortic arch is more important place for the plaque development than abdominal aorta.

## 11.5 Aortic arch

Aortic arch results at 8 month time point showed upregulation of several lipid species due to cigarette smoke exposure. Cholesteryl esters, free cholesterol, ceramides, cerebrosides and globotriasoylceramide species as well as sphingomyelins and phospholipids showed increase in the 3R4F, while this phenomenon was not observed in any other groups. These results are consistent with the previous study (51801 and Boue et al. (Atherosclerosis, 2012)), and also the same trends were observed in C57 mice (51803).

The upregulation in the present study was only observed in the 3R4F group suggesting that P1 does not induce the same changes, and switch or cessation alleviates the changes caused by the 3R4F product. In the previous study in ApoE(-/-) mice cessation or switching to the NGP (P1) product did not decrease the lipid

levels as in the present study. However, it has to be taken into account that in the previous study the mice were exposed for 3 months to 3R4F and 3 months to sham/NGP, whereas in this study the times were 2 and 6 months, respectively. Thus, it is plausible that longer time without 3R4F exposure restores the lipids back to less atherogenic levels.

# 12 ARCHIVING

The signed original Final Report will be made available to the Sponsor and the other original will be archived at the Test Site. All study specific data and documentation will be archived for ten years.

# 13 REFERENCES

[1] H. R. Jung, T. Sylvänne, K. M. Koistinen, K. Tarasov, D. Kauhanen, and K. Ekroos, "High throughput quantitative molecular lipidomics.," *Biochim. Biophys. Acta*, vol. 1811, no. 11, pp. 925–34, Nov. 2011.

[2] M. Ståhlman, C. S. Ejsing, K. Tarasov, J. Perman, J. Borén, and K. Ekroos, "High-throughput shotgun lipidomics by quadrupole time-of-flight mass spectrometry.," *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, vol. 877, no. 26, pp. 2664–72, Sep. 2009.

[3] K. Ekroos, I. V Chernushevich, K. Simons, and A. Shevchenko, "Quantitative profiling of phospholipids by multiple precursor ion scanning on a hybrid quadrupole time-of-flight mass spectrometer.," *Anal. Chem.*, vol. 74, no. 5, pp. 941–9, Mar. 2002.

[4] K. Ekroos, C. S. Ejsing, U. Bahr, M. Karas, K. Simons, and A. Shevchenko, "Charting molecular composition of phosphatidylcholines by fatty acid scanning and ion trap MS3 fragmentation.," *J. Lipid Res.*, vol. 44, no. 11, pp. 2181–92, Nov. 2003.

[5] A. M. McAnoy, C. C. Wu, and R. C. Murphy, "Direct qualitative analysis of triacylglycerols by electrospray mass spectrometry using a linear ion trap.," *J. Am. Soc. Mass Spectrom.*, vol. 16, no. 9, pp. 1498–509, Sep. 2005.

# 14 APPENDICES

APPENDIX 1 (Excel file)
Full data set –Lipid concentrations per sample

APPENDIX 2 (Excel file)
Statistical analyses – groupwise comparisons at each time point

APPENDIX 3 (Excel file)
Statistical analyses – comparisons against 3R4F at 2 month time point

APPENDIX 4 (Excel file)
Statistical analyses – timewise comparisons for each group

APPENDIX 5 (Tableau file)
Full data set with statistics and pathway analysis