# CHAPTER 6

# Mitochondrial dysfunction increases pro-inflammatory cytokine production and impairs regeneration and glucocorticoid responsiveness in lung epithelium

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## Abstract

**Rationale:** Chronic obstructive pulmonary disease (COPD) is a sustained inflammatory disorder of the lungs with progressive functional decline mainly caused by cigarette smoking. The use of lipidomics, an emerging research field, may provide new insights in the onset and progression of chronic inflammatory diseases like COPD.

**Objective:** To investigate whether the expression of the (intra)cellular lipidome is affected by long-term cigarette smoke extract (CSE) exposure in the bronchial epithelial cell line BEAS-2B. Additionally, we studied the mRNA expression of key lipid converting enzymes specific by qPCR.

**Methods:** Cellular changes in lipidome expression was investigated with liquid chromatography and high-resolution quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) in BEAS-2B cells exposed to 0%, 2,5% and 10% CSE for a period of 6 months.

**Measurements and Main Results:** We identified >1500 lipid compounds in BEAS-2B cells. Six-month CSE exposure resulted in 13 up-regulated and 13 down-regulated intracellular sphingolipids in BEAS2B cells. Additionally, the mRNA expression of sphingomyelin synthase 2 and fatty acid synthase was significantly reduced upon long-term CSE exposure.

**Conclusions:** Together, we show an altered lipidome in human bronchial epithelial cells exposed to cigarette smoke for 6 months. Here, mainly lipids from the sphingolipid pathway were affected, providing new insights in our understanding of the role of lipids in cigarette smoke-induced disease.


**Key words:** Lipidomics, Cigarette Smoke Extract, COPD, Airway Epithelial Cells.

## Introduction

Lipidome analysis is an emerging technique that unravels the complex composition of the lipidome of the cell. Lipids play an important role in various cellular processes, including metabolism and energy storage, cellular signaling processes, cell survival and proliferation, cell death and of course membrane integrity (1–4). However, the role of the lipidome in the onset or progression of disease is still poorly understood. Several recent studies have linked the disruption of lipid metabolizing enzymes and pathways to neurodegenerative diseases, cancer and inflammatory diseases (3,5–7), indicating that the deregulation of the lipidome may play a role in their pathogenesis. Chronic obstructive pulmonary disease (COPD) is a sustained lung inflammatory disease affecting millions of people worldwide. In the industrialized world, long-term cigarette smoking is the main risk factor for COPD, in combination with a genetic predisposition (8,9). In an explorative study, we have previously shown that lipidome profiles in sputum differ between healthy non-smokers, smokers without COPD and smokers with COPD. Lipid quantity of over 200 individual lipid components, covering four major lipid classes of the sphingolipid pathway, i.e. ceramids, sphingomyelins, glycosphingolipids and ethanolamines, differed between smokers without COPD and smokers with COPD (1). However, in this study we could not specify which cell type contributed to these changes nor could we determine (intra)cellular lipidome changes. As the airway epithelium is the first line of defense against noxious particles such as cigarette smoke, epithelial cells are thought to contribute to the aberrant inflammatory response to cigarette smoking in COPD (8,10–15). The lipidome may play a role in the epithelial pro-inflammatory response to cigarette smoke, as pro-inflammatory cytokines are known to affect the activity of lipid-modifying/metabolizing enzymes, such as phospholipases, sphingomyelinases and sphingosine kinases, leading to changes in lipids that are known to regulate inflammatory signaling (1,6).

We hypothesize that the lipidome may also be affected directly by cigarette smoking. We have previously shown that long-term cigarette exposure of human airway epithelium impairs the structure and function of mitochondria, which are lipid rich organelles important for energy exchange (12). To assess whether cigarette smoking induces alterations in the lipidome, we investigated long-term cigarette smoke exposure in human bronchial epithelial BEAS-2B cells. We studied the effect of 6-month exposure to 2.5% and 10% cigarette smoke extract (CSE) on lipid expression and sphingomyelinase activity in the human bronchial cell line BEAS-2B *in vitro*. Additionally, gene expression of 6 enzymes that regulate lipid metabolism, Sphingomyelin synthase 1 and 2 (SGMS1 and 2), fatty acid synthase (FASN), sphingomyelin phosphodiesterase 1 (SMPD1), neutral sphingomyelinase activation associated factor (NSMAF) and ceramide kinase (CERK) was quantified by reverse-transcription PCR (qRT-PCR).

## Methods and materials

### In vitro study

BEAS-2B cells were grown in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 15% heat-inactivated Fetal Bovine Serum (FBS), penicillin (100 U/ml) (Lonza, Verviers, Belgium) and streptomycin (100 µg/ml) (Lonza, Verviers, Belgium) on collagen-coated plates and 0%, 2.5% and 10% CSE. Cells were grown to ~90 % confluence and passaged twice a week by trypsin. Cigarette smoke extract (CSE) was prepared from filter-less research-grade cigarettes (3R4F, Tobacco Research Institute, University of Kentucky, Lexington, KY) as described previously (16). Smoke from 2 cigarettes was bubbled through 25 ml medium was considered as 100% CSE. CSE was added freshly each time when the cells were passaged.

### Airway epithelial (BEAS-2B) cell lipid extraction

Once cells were grown to ~ 90% confluence, medium was replaced by serum deprived medium (containing 0 2.5 or 10% CSE) 24 hours before the experiment. Methanol was used to extract the lipids by direct application to the mono-layer cell culture. 200 µL aliquots of methanol extracts were transferred into 500 µL Eppendorf tubes and centrifuged at 15,000 rpm during 10 min. The resulting supernatant (185 µL) was transferred into a vial with insert, dried in centrifugal vacuum evaporator (miVac duoconcentrator, Genevac Lim., Ipswich, UK) and reconstituted in 37 µL chloroform/isopropanol 50/50 (v/v). 10 µL of this extract was injected into the LC-MS system

### Lipidomics analysis, experimental setup, preparation and use of quality control (QC) samples

For each experiment, the use of QC samples allowed monitoring the repeatability and stability of the lipid measurement. BEAS-2B cell lines were investigated after 6-month exposure to cigarette smoke extract (CSE) (15 cell extracts, 6 QCs). To prepare QC samples within each experiment, 60 µL aliquots of the samples were collected in a QC pool. This QC pool was then divided into 120 µL aliquots to obtain representative QC samples. QC samples were prepared simultaneously along with study samples and were analyzed throughout the LC/MS analysis sequences every five study samples. These samples did not contain any biological variability and can be considered as technical replicates. The relative standard deviation percent (RSD%) of all significantly up- or downregulated lipids has been calculated to demonstrate the measurement error of the lipidomics analytical platform. A more detailed description of the lipidomic analysis is available in the supplement.

### Lipid Nomenclature

For this study the International Lipid Classification and Nomenclature Committee (ILCNC) was used, i.e. "Comprehensive Classification System for Lipids" (17–19). Regarding glycosphingolipids, LC-MS is not able to discriminate between galactose and glucose, or N-acetylglucosamine and N-acetylgalactosamine. Therefore, lipid nomenclature of these species contains hexose (Hex) instead of glucose or galactose.

### qRT-PCR

RNA was extracted from homogenates from the long-term exposed cells using TriReagent (Applied Biosystems/Ambion, Foster City, CA, USA), reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Herculus, CA, USA). Sphingomyelin synthase 1 and 2 (SGMS1 and 2), fatty acid synthase (FASN), sphingomyelin phosphodiesterase 1 (SMPD1), neutral sphingomyelinase activation associated factor (NSMAF) and ceramide kinase (CERK) expression was analyzed by real-time PCR using Taqman® (Applied Biosystems, Foster City, CA, USA). Validated probes and housekeeping genes, B2M and PP1A and TaqMan Master Mix were purchased from Applied Biosystems (Foster City, CA, USA).

### Statistical analyses

Differences in expression levels were compared with a Mann-Whitney U test for all comparison experiments. Multiple testing correction, according to the false discovery rate, was performed for the results described in table 1 and table 2 (20). Compounds that were significantly differentially expressed ($p<0.05$) were identified with tandem mass spectrometry (MS/MS).



## Results

### Long-term exposure of BEAS-2B cells to CSE

We have previously shown that 6-month CSE exposure alters mitochondrial morphology and function in the bronchial epithelial cell line BEAS-2B (22). Therefore, we first tested whether 6-months exposure to 0% (control), 2.5% and 10% CSE also affects the lipidome in BEAS-2B cells. Table 1 shows all lipid compounds that were detected in BEAS-2B cells grown for 6 months without CSE. No significant changes were observed when the cells were grown for 6 months in a low concentration of CSE (2.5%), although a trend towards either up-regulation or down-regulation was visible for 12 sphingolipids (Table 1), including 2 ceramides, 1 dihydroceramide, 3 sphingomyelins, and 6 Glycoshingolipids GSLs (Table 1). The high concentration of CSE (10%) significantly altered the expression of 26 sphingolipids: 4 ceramides, 1 dihydroceramide, 9 sphingomyelins and 12 GSLs (Table 2).

**Table 1:** Differentially regulated lipids after long-term exposure to low CSE concentration (2.5%)

| Class | Subclass | Lipid | Molecular structure | Fold change | *p*-value | RSD QC (%) |
|---|---|---|---|---|---|---|
| ceramides | ceramides | Cer(d18:1/14:1) | $C_{32}H_{61}NO_3$ | 0.19 | 0.067 | 8.22 |
| | | Cer(d18:1/22:1) | $C_{40}H_{77}NO_3$ | 0.35 | 0.067 | 5.36 |
| | dihydroceramides | Cer(d18:0/14:0) | $C_{32}H_{65}NO_3$ | 0.55 | 0.067 | 11.93 |
| sphingomyelins | | SM(d18:1/14:0) | $C_{37}H_{75}N_2O_6P$ | 0.75 | 0.098 | 9.25 |
| | | SM(d18:1/14:1) | $C_{37}H_{73}N_2O_6P$ | 0.57 | 0.067 | 7.96 |
| | | SM(d18:1/24:2) | $C_{47}H_{91}N_2O_6P$ | 0.54 | 0.067 | 9.17 |
| glycosphingolipids (2 sugars) | dihexosyl-ceramides | Hex-HexCer(d18:1/18:0) | $C_{48}H_{91}NO_{13}$ | 2.13 | 0.067 | 4.09 |
| | | Hex-HexCer(d18:1/24:0) | $C_{54}H_{103}NO_{13}$ | 1.80 | 0.067 | 6.49 |
| glycosphingolipids (other) | N-acetylhexosamine-trihexosyl-ceramides | HexNAc-Hex-Hex-HexCer(d18:1/24:0) | $C_{68}H_{126}N_2O_{23}$ | 7.32 | 0.098 | 8.36 |
| | neuraminic acid containing dihexosyl-ceramides | NeuAc-Hex-HexCer(d18:1/16:0) | $C_{57}H_{104}N_2O_{21}$ | 0.76 | 0.299 | 5.56 |
| | | NeuAc-Hex-HexCer(d18:1/24:0) | $C_{65}H_{120}N_2O_{21}$ | 0.50 | 0.098 | 4.08 |
| | | NeuAc-Hex-HexCer(d18:1/24:1) | $C_{65}H_{118}N_2O_{21}$ | 0.74 | 0.156 | 9.12 |

Fold change = expression in exposed cells / in non-exposed cells. A fold change >1 is up-regulation in exposed cells. A fold change between 0 and 1 is down-regulation in exposed cells. The LipidMaps database was taken as the reference for the lipid nomenclature. LC-MS cannot discriminate between galactose and glucose, or N-acetylglucosamine and N-acetylgalactosamine. E.g. LacCer can also be Gal-GalCer. Therefore, lipid nomenclature contains hexose (Hex) instead of glucose or galactose. Relative standard deviation (RSD%) has been calculated from the QC samples (n=6).

**Table 2:** Differentially regulated lipids after long-term exposure to high CSE concentration (10%)

| Class | Subclass | Lipid | Molecular structure | Fold change | *p*-value | RSD QC (%) |
|---|---|---|---|---|---|---|
| ceramides | ceramides | Cer(d18:1/16:0) | $C_{34}H_{67}NO_3$ | 2.42 | 0.028 | 10.19 |
| | | Cer(d18:1/17:0) | $C_{35}H_{69}NO_3$ | 3.52 | 0.028 | 14.18 |
| | | Cer(d18:1/14:1) | $C_{32}H_{61}NO_3$ | 0.08 | 0.028 | 8.22 |
| | | Cer(d18:1/22:1) | $C_{40}H_{77}NO_3$ | 0.22 | 0.028 | 5.36 |
| | dihydroceramides | Cer(d18:0/14:0) | $C_{32}H_{65}NO_3$ | 0.47 | 0.028 | 11.93 |
| sphingomyelins | | SM(d18:0/14:0) | $C_{37}H_{77}N_2O_6P$ | 0.43 | 0.028 | 7.93 |
| | | SM(d18:1/12:0) | $C_{35}H_{71}N_2O_6P$ | 0.33 | 0.028 | 10.94 |
| | | SM(d18:1/14:0) | $C_{37}H_{75}N_2O_6P$ | 0.66 | 0.028 | 9.25 |
| | | SM(d18:1/16:0) | $C_{39}H_{79}N_2O_6P$ | 1.37 | 0.028 | 5.86 |
| | | SM(d18:1/17:0) | $C_{40}H_{81}N_2O_6P$ | 2.32 | 0.028 | 4.84 |
| | | SM(d18:1/14:1) | $C_{37}H_{73}N_2O_6P$ | 0.37 | 0.028 | 7.96 |
| | | SM(d18:1/16:1) | $C_{39}H_{77}N_2O_6P$ | 0.50 | 0.028 | 11.08 |
| | | SM(d18:1/18:1) | $C_{41}H_{81}N_2O_6P$ | 0.43 | 0.041 | 4.81 |
| | | SM(d18:1/24:2) | $C_{47}H_{91}N_2O_6P$ | 0.36 | 0.028 | 9.17 |
| glycosphingolipids (2 sugars) | dihexosyl-ceramides | Hex-HexCer(d18:1/16:0) | $C_{46}H_{87}NO_{13}$ | 2.48 | 0.028 | 6.81 |
| | | Hex-HexCer(d18:1/24:0) | $C_{54}H_{103}NO_{13}$ | 2.58 | 0.041 | 6.49 |
| glycosphingolipids (3 sugars) | trihexosyl-ceramides | Hex-Hex-HexCer(d18:1/16:0) | $C_{52}H_{97}NO_{18}$ | 2.67 | 0.028 | 6.69 |
| | | Hex-Hex-HexCer(d18:1/20:0) | $C_{56}H_{105}NO_{18}$ | 2.67 | 0.041 | 9.57 |
| | | Hex-Hex-HexCer(d18:1/24:0) | $C_{60}H_{113}NO_{18}$ | 2.09 | 0.041 | 4.40 |

**Table 2:** Continued

| Class | Subclass | Lipid | Molecular structure | Fold change | *p*-value | RSD QC (%) |
|---|---|---|---|---|---|---|
| glycosphinogolipids (other) | N-acetylhexosamine-trihexosyl-ceramides | HexNAc-Hex-Hex-HexCer(d18:1/24:1) | $C_{68}H_{124}N_2O_{23}$ | 9.07 | 0.028 | 11.17 |
| | | HexNAc-Hex-Hex-HexCer(d18:1/16:0) | $C_{60}H_{110}N_2O_{23}$ | 6.63 | 0.028 | 10.41 |
| | | HexNAc-Hex-Hex-HexCer(d18:1/22:0) | $C_{66}H_{122}N_2O_{23}$ | 3.45 | 0.028 | 15.65 |
| | | HexNAc-Hex-Hex-HexCer(d18:1/24:0) | $C_{68}H_{126}N_2O_{23}$ | 8.89 | 0.028 | 8.36 |
| | neuraminic acid containing dihexosyl-ceramides | NeuAc-Hex-HexCer(d18:1/16:0) | $C_{57}H_{104}N_2O_{21}$ | 0.54 | 0.041 | 5.56 |
| | | NeuAc-Hex-HexCer(d18:1/24:0) | $C_{65}H_{120}N_2O_{21}$ | 0.48 | 0.028 | 4.08 |
| | | NeuAc-Hex-HexCer(d18:1/24:1) | $C_{65}H_{118}N_2O_{21}$ | 0.56 | 0.028 | 9.12 |

Fold change = expression in exposed cells / in non-exposed cells. A fold change >1 is up-regulation in exposed cells. A fold change between 0 and 1 is down-regulation in exposed cells. The LipidMaps database was taken as the reference for the lipid nomenclature. LC-MS cannot discriminate between galactose and glucose, or N-acetylglucosamine and N-acetylgalactosamine. E.g. LacCer can also be Gal-GalCer. Therefore, lipid nomenclature contains hexose (Hex) instead of glucose or galactose. Relative standard deviation (RSD%) has been calculated from the QC samples (n=6). -

Neuraminic acid containing sphingolipids, ceramides and sphingomyelins were all down-regulated, except for 2 specific ceramides and their related sphingomyelins (i.e. d18:1/16:0 and d18:1/17:0 species). In contrast, other glycosphingolipids were significantly up-regulated in cells exposed to 10% CSE (3-9 fold), with the strongest effect on N-acetylhexosamine-trihexosyl-ceramides. Of note, all lipids that tended to increase or decrease in the 2.5% CSE-exposed cells were significantly differentially regulated in 10% CSE-exposed cells in the same direction.

Additionally, we investigated the effect of long-term CSE exposure on sphingomyelinase activity by assessing the ratio of ceramides and sphingomyelin species with identical carbon chain length and saturation (21). Here, a higher ratio indicates that more sphingomyelin is converted to ceramides, reflecting a higher sphingomyelinase activity. The ceramide to sphingomyelin ratio for d18:1/16:0 was increased both in 2.5% and 10% CSE-exposed BEAS-2B cells, while the ratio of d18:1/17:0 and d18:1/18:0 was only significantly increased in BEAS-2B exposed to 10% CSE (Figure 1).

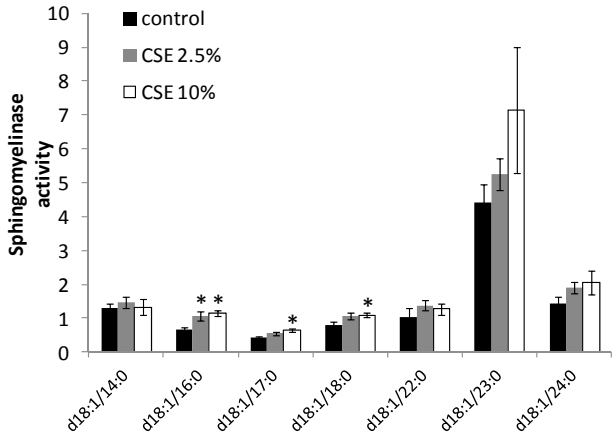


**Figure 1:** Sphingomyelinase activity in BEAS.2B cells (mean+/-SEM). Sphingomyelinase activity was calculated as the ratio between a ceramide and its related sphingomyelin, with identical sphingoid base and fatty acid chain, e.g. Cer(d18:1/16:0) and SM(d18:1/16:0). Sphingomyelinase activity was increased for d18:1/16:0 (2.5% and 10% CSE), d18:1/17:0 (10% CSE) and d18:1/18:0 (10% CSE) in CSE exposed BEAS.2B cells. AUC = area under the curve. * = $p<0.05$ in comparison with control

## qRT-PCR of lipid modifying enzymes in BEAS-2B cells exposed to 0%, 2.5% and 10% CSE for 6 months

To uncover which lipid modifying enzymes are affected by cigarette smoke and may be responsible for the observed lipidome alterations, we investigated the transcriptional levels of sphingomyelin synthase 1 and 2 (SGMS1 and 2), fatty acid synthase (FASN), sphingomyelin phosphodiesterase 1 (SMPD1), neutral sphingomyelinase activation associated factor (NSMAF) and ceramide kinase (CERK) in BEAS-2B cells exposed to 0%, 2.5% and 10% CSE for 6 months. SGMS2 transcription was significantly lowered by 2.5% and more strongly by 10% CSE (Figure 2). In contrast, SGMS1 was not significantly altered by 2.5% nor by 10% CSE. Like SGMS2, transcriptional levels of FASN were already lowered by 2.5% CSE exposure and further reduced by 10% CSE. SMPD1 was slightly reduced by the highest concentration of 10% CSE, although this failed to reach statistical significance (p=0.0556). There was no significant difference in transcriptional levels of NSMAF and CERK. These data show that long-term CSE exposure alters the lipid profile in human bronchial epithelial cells and that alterations in expression of FASN and SGMS2, but not SGMS1, may be responsible for the observed changes.

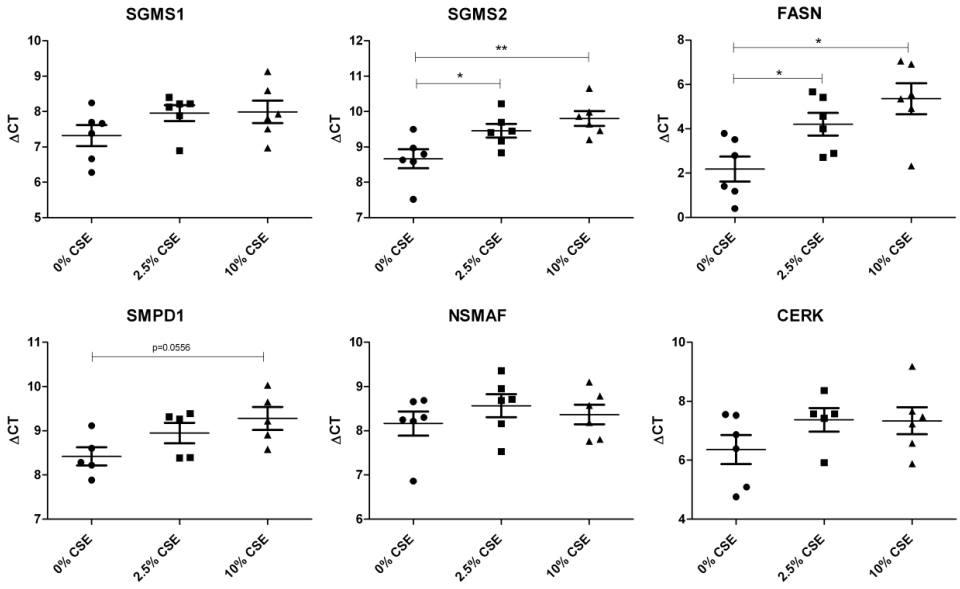


**Figure 2:** qRT-PCR on long-term cigarette smoke exposed cells, 0% CSE, 2.5% CSE and 10%CSE, show a significant decrease in transcriptional levels of FASN, SGMS2 in 2.5% CSE and 10% CSE when compared to control conditions. A decreasing trend is visible for SMPD1 in cells exposed to 10% CSE when compared to control conditions. No significant changes in transcriptional levels were found for SGMS1, NSMAF and CERK. SGMS = sphingomyelin synthase; FASN = fatty acid synthase; SMPD1 = sphingomyelin phosphodiesterase 1; NSMAF = neutral sphingomyelinase activation associated factor; CERK = ceramide kinase (CERK)

## Discussion

We hypothesized that cigarette smoke affects epithelial expression of lipids that are relevant to the pathogenesis of COPD, regulating inflammatory signaling (1,6). In the current study, we show that lipids from the sphingolipid pathway are altered by long-term CSE exposure in human bronchial epithelial cells, which is in line with our previous study showing that the same lipids are down-regulated in sputum of smokers with and without COPD compared to non-smokers (1). This suggests that lipids derived from airway epithelial cells may be responsible for the observed changes in the lipid profile in sputum upon long-term smoking. In our previous study, we found that all lipid components were increased in smokers compared to non-smokers, except for neuraminic acid containing lipids, which was down-regulated. Accordingly, we observed a decrease in three neuraminic acid containing lipid components belonging to the glycosphinogolipids, i.e. sphingolipids, ceramides and sphingomyelins. One identical component, NeuAc-Hex-HexCer(d18:1/24:1), was observed to be elevated in smokers compared to non-smokers. Also, a second identical neuraminic acid containing component, NeuAc-Hex-HexCer(d18:1/24:0), was observed to be increased in COPD patients compared to healthy smokers. On the other hand, we observed an increase in other glycosphinogolipids, especially lipids belonging to N-acetylhexosamine-trihexosyl-ceramides. This is again in line with our previous findings, showing higher levels of the same lipid class in sputum from smokers and especially in COPD (1). Two of the lipid components increased by 10% CSE exposure, HexNAc-Hex-Hex-HexCer(d18:1/16:0) and HexNAc-Hex-Hex-HexCer(d18:1/22:0), were also observed to be increased in sputum from COPD patients compared to healthy smokers. Furthermore, we observed that long-term cigarette smoke exposure significantly reduced the expression of the lipid converting enzymes SGMS2 and FASN in bronchial epithelial cells, which may account for the observed changes in lipid profile. The changes in lipidomic profile and regulating enzymes suggest that cigarette smoke affects the expression of key components regulating lipid expression. Indeed, sphingomyelinase activity, as determined by the ratio of ceramides and sphingomyelin, was increased upon long-term CSE exposure for ceramides and sphingomyelins with a fatty acid chain length of 16 to 18 carbon atoms. SGMS1 and SGMS2 catalyze the conversion of phosphatidylcholine and ceramides to sphingomyelin (SM) and diacylglycerol. This is in line with a recent publication showing that neutral sphingomyelinase 2 (nSMase2) is the sole sphingomyelinase being activated during cigarette smoke-induced oxidative stress and where src acts as a regulator in human airway epithelial cells (23). Thus, the observed reduction in SGMS2 expression upon long-term cigarette smoke exposure may be responsible for the increased sphingomyelinase activity. However, most sphingomyelins, 7 of 9, were down-regulated of which 5 components were identical to the increased sphingomyelins found in COPD patients. These components include the only 2 increased sphingomyelins, SM(d18:1/16:0) and SM(d18:1/17:0), found in 10% CSE exposed BEAS-2B

cells. The conversion of phosphatidylcholine and ceramides is a process required for cell growth and membrane stability. Sphingomyelin plays a key role in apoptosis regulation (24–27) and alterations in its levels in airway epithelium may thus have consequences for COPD (16,26,27). We also observed that long-term CSE exposure reduces mRNA expression of FASN, a multifunctional enzyme that catalyzes the formation of long-chain fatty acids from one of the most ubiquities lipid component, palmitate. Reduced FASN expression has also been observed under hypoxia induced cell death conditions in hepatocytes (28), whereas cigarette smoke augments hypoxic chemo-sensitivity in humans which may lead to accelerated cell death (29). The observed reduction in FASN expression may also explain the reduction in various long-chain lipid components, as the synthases of these lipids may be hampered in bronchial epithelial cells upon smoke exposure. Thus, our findings on the mRNA expression of FASN and SGM2 are in line with the observed changes in lipid profile, although future studies will be required to confirm that protein levels of these enzymes are also reduced upon long-term CSE exposure.

The reduction in neuraminic acid containing lipid components that was observed *in vitro* as well as in sputum of smokers and COPD patients may have important implications for the development of the disease. Neuraminic acids are terminal monosaccharides modifications of most glycoconjugates and glycosphingolipids. Of these, N-acetylneuraminic acid is involved in a variety of biological interactions including cell adhesion and migration (30), thereby regulating damage and repair processes. Ceramides are known to affect mitochondrial membrane permeability and induce the formation of membrane channels, which in turn, may release pro-apoptotic proteins from the mitochondria (31–33). Thus, the observed cigarette smoke-induced chances may have important implications for mitochondrial function and cell survival, which is in line with our previous findings on the effects of cigarette smoke on mitochondrial structure and function (12). Also, oxidative stress and inflammatory stimuli were shown to induce ceramide generation, which has been linked to the development and progression of COPD disease (26,27,34–36). Two ceramides, Cer(d18:1/16:0) and Cer(d18:1/17:0), were increased in BEAS-2B cells exposed to 10% CSE. Both lipid components were also observed to be elevated in sputum from COPD patients when compared to healthy smokers. The release of ceramides can induce cellular apoptosis and cell death (6,26,27,37). In contrast, 3 other ceramides were actually down regulated (of which 2 of them were actually increased in lung sputum of COPD patients) in BEAS-2B cells exposed to 10% CSE. This might suggest a role for ceramides in possibly a biological signaling response to cigarette smoke induced stress (37,38).

In conclusion, our findings show that long-term CSE exposure induces up and down-regulation of lipids belonging to the same class of lipids that were up or down-regulated in induced sputum of smokers with and without COPD. This demonstrates again that LC-QTOF-MS(/MS) LC-MS with MS/MS is also a powerful tool to identify lipids profiles *in*

*vitro*. Lipidome profiling may unravel cigarette-smoke induced changes important for the pathogenesis of COPD.

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