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应用纪要

Quantifying the Lipidome for a Respiratory Disease Study Using LipidQuan: A Rapid and Comprehensive Targeted Approach

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Abstract

This application note describes a rapid quantitative method (LipidQuan) used to analyze the plasma from patients diagnosed with COPD and asthma in comparison to healthy controls

Benefits

- Rapid quantification of over 500 lipids in plasma/serum, covering a large dynamic range
- A robust and easy to deploy platform that uses Quanpedia to reduce method development time and training costs
- Improve identification and specificity of phospholipids using MRM transitions from the two fatty acyl chain fragments
- Flexible, fast data processing and visualization using TargetLynx Software and third party informatics (i.e. Skyline)

- Faster and cheaper than comparable workflows, increasing productivity

Introduction

Chronic obstructive pulmonary disease (COPD) represents a group of lung conditions, including emphysema and chronic bronchitis, that result in significant breathing impairment (Figure 1). COPD is a non-curable disease that affects 251 million people worldwide and resulted in 3.17 million deaths in 2015 (WHO, 2017). The main cause of COPD is exposure to tobacco smoke and primarily effects middle-aged or older adults with a smoking history. Asthma and pulmonary infection are conditions linked to COPD and are driving significant increases in socio-economic cost. Recent reports have shown, for example, that the costs associated with treatment of COPD related conditions will exceed £11 billion per year in the UK.[1] In addition to lifestyle factors such as smoking, molecular factors are known to increase the probability of developing the condition. Previous studies have shown altered lipid profiles are associated with the emergence and propagation of COPD.[2,3] Although advances in mass spectrometry (MS) have allowed for more in-depth lipidomic analysis, unambiguous identification and quantification has proven difficult as lipids exhibit a high number of isomeric and isobaric species. Here, we describe the utilization of the LipidQuan[4] workflow (Figure 2) to overcome these challenges and to provide a comprehensive and quantitative assessment of the lipid species involved in COPD and asthma.

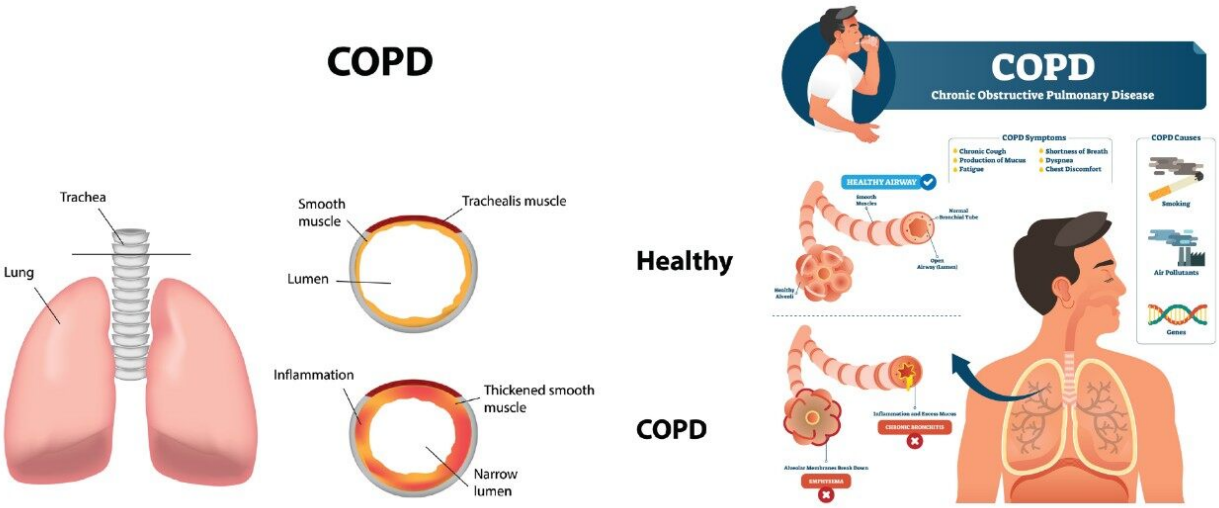

Figure 1. COPD results in restricted airways and damaged alveoli leading to poor lung function and breathing difficulties.

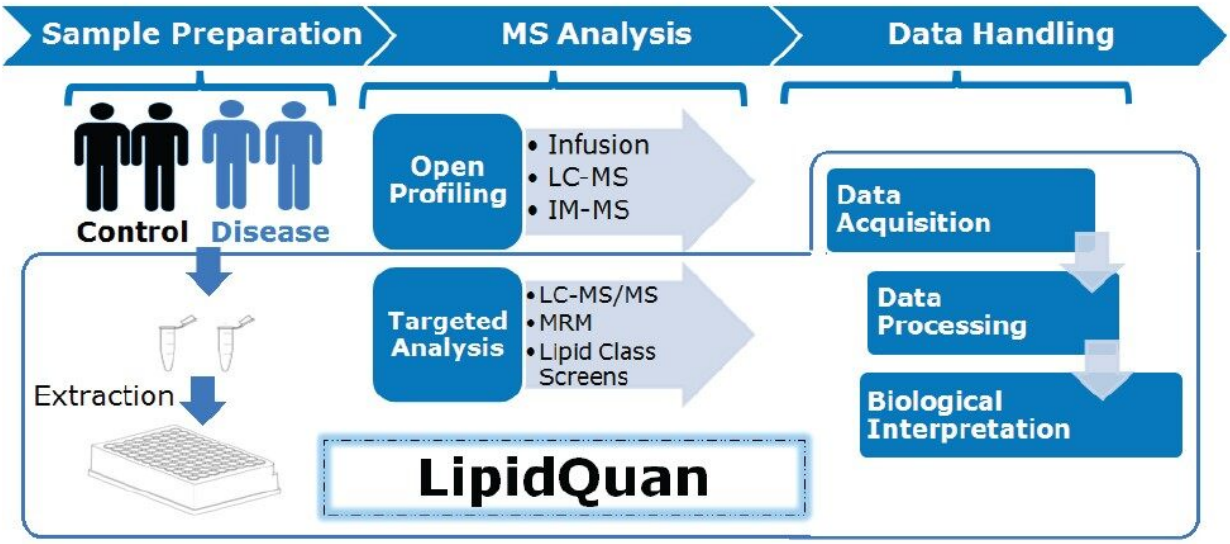

Figure 2. General lipidomic workflow used in most research laboratories with the LipidQuan workflow highlighted.

Experimental

Samples

The study analyzed plasma samples from 21 individuals: healthy controls healthy controls (n=6), COPD (n=9), and asthma (n=6).

Stable isotope labelled (SIL) standards (SPLASH LIPIDOMIX, Avanti Lipids, Alabaster, AL) spiked into pooled human plasma were used to generate calibration curves for quantification. In total, nine concentration levels were constructed representing all the lipid classes contained within the SIL standard. High, Middle, and Low QC samples were also prepared at 80%, 8%, and 1.275%, respectively of the highest concentration of the calibration curve.

Sample preparation

A simple sample preparation procedure was adopted using protein precipitation with pre-cooled isopropanol (IPA) spiked with the odd Chain LIPIDOMIX (Avanti Lipids, Alabaster, AL), (1:5, plasma:IPA). Samples were vortex mixed for one minute and placed at -20 °C for 10 minutes. Samples were vortex mixed again for one minute and placed at 4 °C for two hours to ensure complete protein precipitation. The extracted samples were centrifuged at 10,300 g for 10 minutes at 4 °C and the supernatant was transferred to glass vials for LC-MS/MS analysis. Prepared samples were analyzed in duplicate for both positive and negative ionization modes.

LC conditions

LC system:	ACQUITY UPLC I-Class or H-Class (Fixed Loop (FL) or Flow Through Needle (FTN))
Column(s):	ACQUITY UPLC BEH Amide 2.1 × 100 mm, 1.7 µm (p/n: 186004808)
Column temp.:	45 °C
Flow rate:	0.6 mL/min
Mobile phase:	95:5 Acetonitrile/water + 10 mM Ammonium acetate (A) and 50:50 Acetonitrile/water + 10 mM Ammonium acetate (B)

Gradient:	0.1% to 20.0% B for 2 minutes, then 20% to 80% B for 3 minutes followed by 3 minutes re-equilibration
Run time:	8 minutes
Injection volume:	2 µL

MS conditions

MS systems:	Xevo TQ-XS, TQ-S, or TQ-S micro
Ionization mode:	ESI (+/-)
Capillary voltage:	2.8 kV (+) 1.9 kV (-)
Acquisition mode:	MRM
Source temp.:	120 °C
Desolvation temp.:	500 °C
Cone gas flow:	150 L/hr
Desolvation flow:	1000 L/hr
Nebulizer gas:	7 bars
Ion guide offset 1:	3 V
Ion guide offset 2:	0.3 V

Informatics

A downloadable LipidQuan Quanpedia method file that contains the LC conditions, the MS method, and the associated TargetLynx processing method (including retention times and MRM transitions)

was used. The resulting data were processed with either TargetLynx or Skyline (MacCoss Lab Software, University of Washington). Statistical analysis was performed using SIMCA P+ (Umetrics, Umeå, Sweden) and further data interrogation conducted using Metaboanalyst.[5]

Results and Discussion

Plasma samples from three biological states of varying phenotype (healthy control, COPD patients, and asthma patients) were analyzed. Samples were prepared using a simple protein precipitation with cooled IPA before targeted LC-MS/MS data were acquired in positive and negative ion electrospray modes using the LipidQuan platform. Samples were randomized and two technical replicates per sample were acquired. Example chromatograms representing endogenous lipids for both positive and negative ion mode are shown in Figure 3. A large number of lipids were identified from the extensive LipidQuan MRM library, a library that contains highly specific fatty acyl transitions and head group fragments for increased identification confidence. Figure 4 shows the power of the LipidQuan MRM library. In this example, phosphocholines (PC), which have two potential identifications from the same precursor mass, were distinguished by the specific fatty acyl chain fragments.

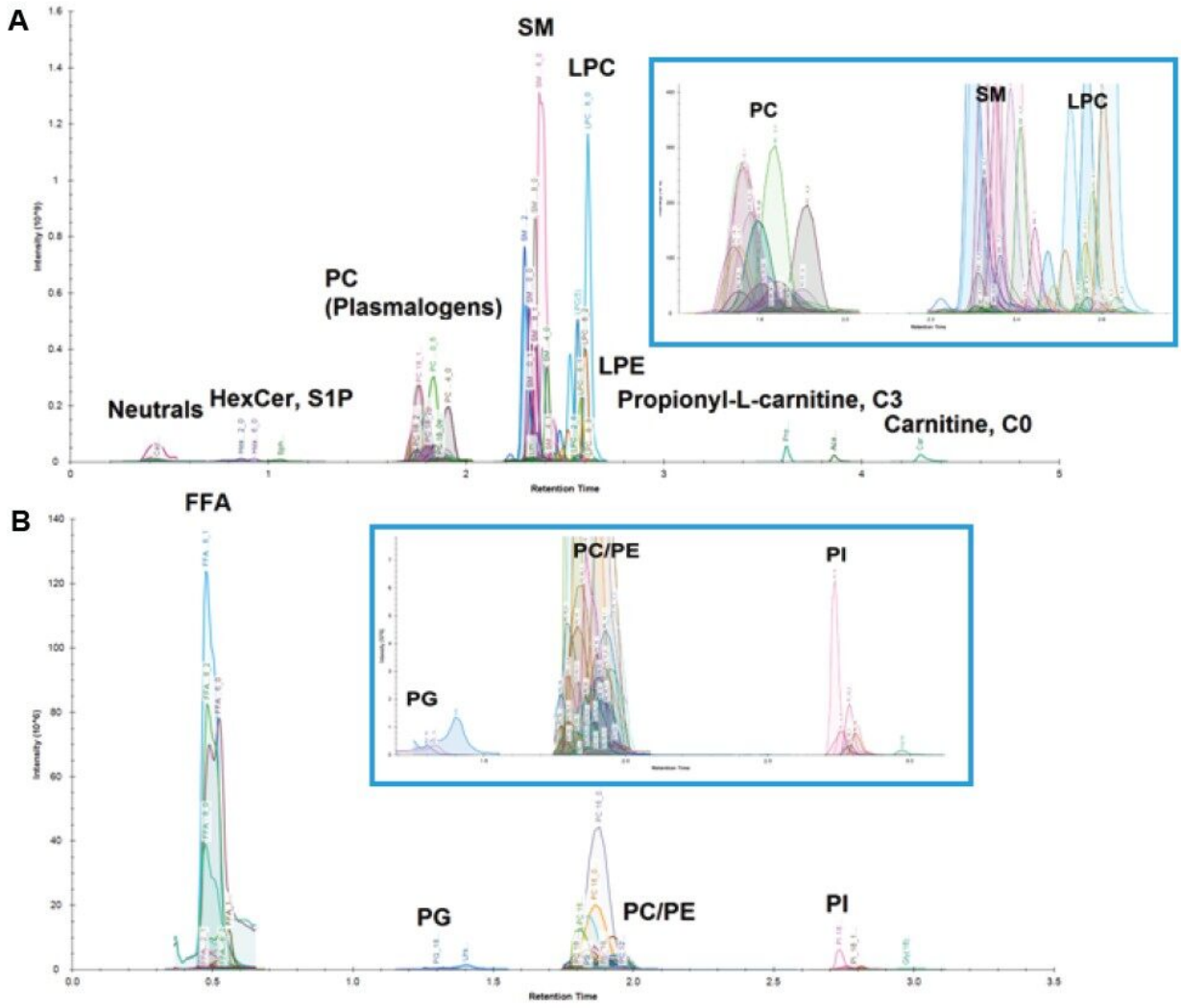

Figure 3. Overlaid chromatograms representing Positive Mode (A) and Negative Mode (B) plasma screen.

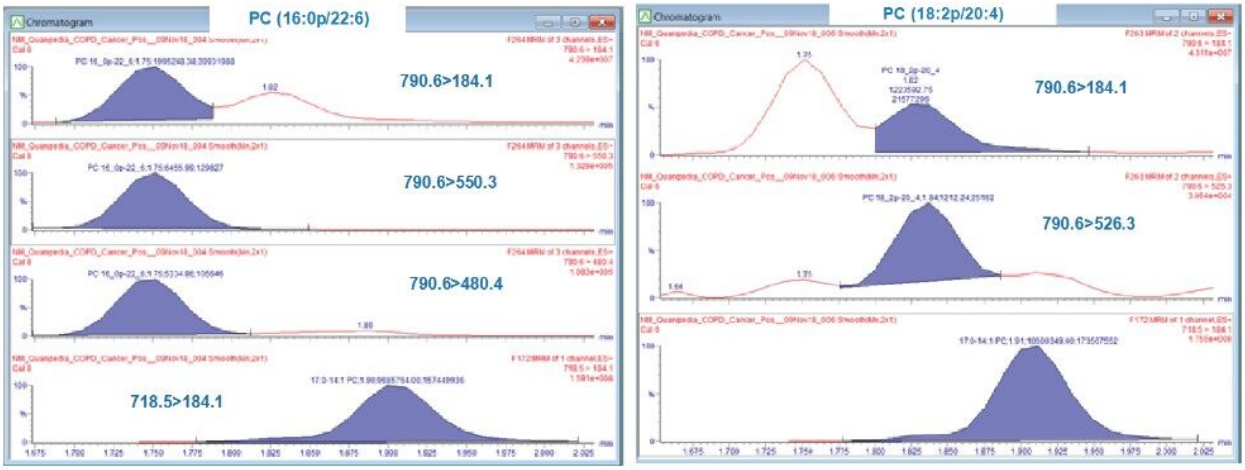

Figure 4. LipidQuan improves isobaric lipid identification by using fatty acyl fragment transitions and retention times for confirmation. For example, PC (16:0p/22:6), and PC (18:2p/20:4) share the same precursor m/z 790.6 and therefore cannot be distinguished using only the head group transition (m/z 184.1). However, these PCs are distinguished by their fatty acyl chain fragment transitions.

Statistical analysis of the data revealed clear separation between the various cohorts (Figure 5). Validated PLS-DA models resulted in the clustering of healthy controls, COPD, and asthma patients (Figure 5A). Additional validation, through the use of permutation tests, indicated that the applied PLS-DA models did not over fit the data (R^2=0.843, Q2=0.844). The loadings plot (Figure 5B), indicated that FFAs, PCs, LPCs, SMs, and Ceramides are the main contributors of statistical separation. Additional curation of the data using ANOVA/t-test revealed the 100 most statistically significant lipids for hierarchical clustering, showing differential lipid expression trends for the three cohorts (Figure 5C).

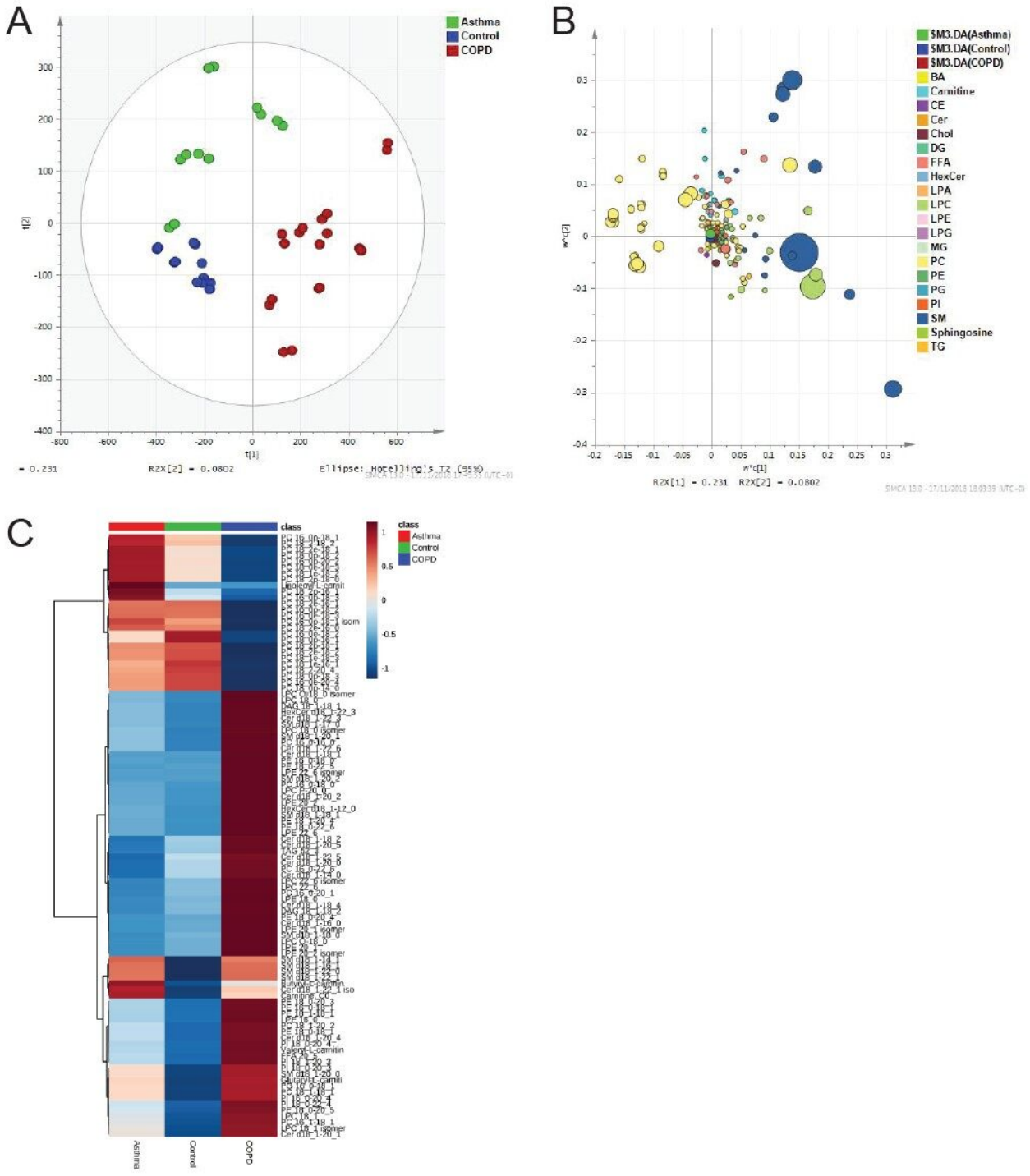

Figure 5. Multivariate statistical analysis shows distinct group separation between the three patient groups as demonstrated by the PLS-DA (pareto scaling) plot (A). The corresponding loadings plot (B) indicates the contribution of the various lipid classes to the separation observed with the PLS-DA model. (NB: the size of the plot marks will be smaller or larger than the default plot mark depending on the value for the specific

observation or variable in the selected vector.) ANOVA/t-test with 1% FDR thresholding results in the top 100 lipid identifications. Hierarchical clustering of these 100 lipids highlights the averaged differential expression across the three groups (C).

Quantification was achieved using calibration curves of plasma spiked with known concentrations of SIL standards prior to extraction. Further details of calibration ranges and acceptance criteria can be found in the LipidQuan Method Reference Guide (www.waters.com/targetedomics). Using surrogate standards prepared and analyzed under identical conditions as the control and disease samples, the quantification of endogenous lipids within the same class was achieved. Deuterated standards were used to assess linear response, with typical R^2 values ranging from 0.97–0.99 for the various lipid classes in both modes of ionization. Figure 6 shows example calibration curves for LPC, PC, and PE species.

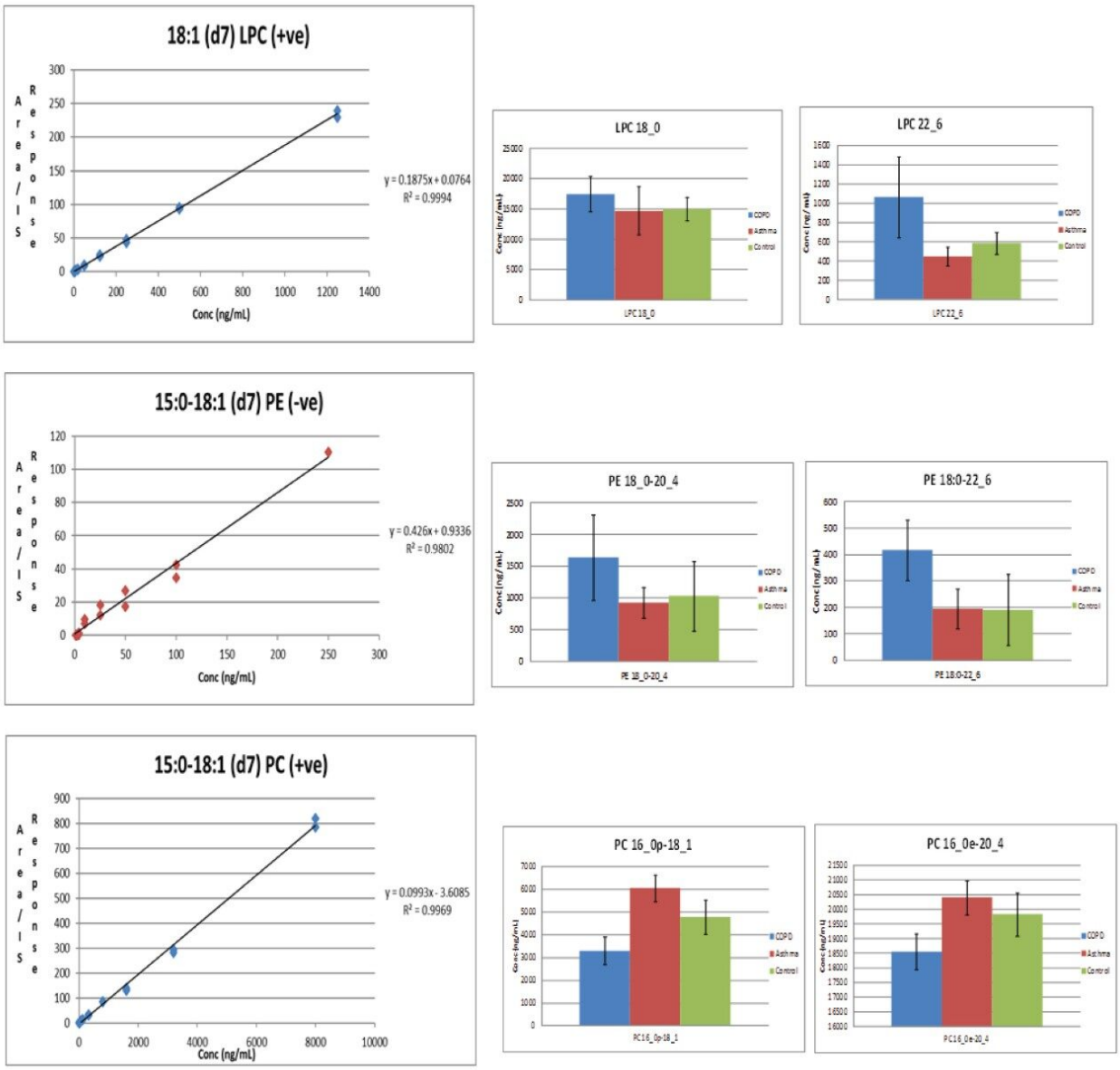

Figure 6. Quantitative data representing statistical significant lipid species (ANOVA/t-test with an FDR <1%) for over expressed LPC (A), PE (B), and down regulated PC (C).

Biological relevance was explored by merging the data from all experiments and performing pathway analysis. This analysis revealed a correlation of phenotype with altered inflammatory, oxidative, and immunity processes and suggests the involvement of differential regulation of specific lipid species in signalling, metabolic, and regulatory pathways. Lipoprotein metabolism, for example, was identified as a pathway that is highly dysregulated in the COPD phenotype (Figure 7).

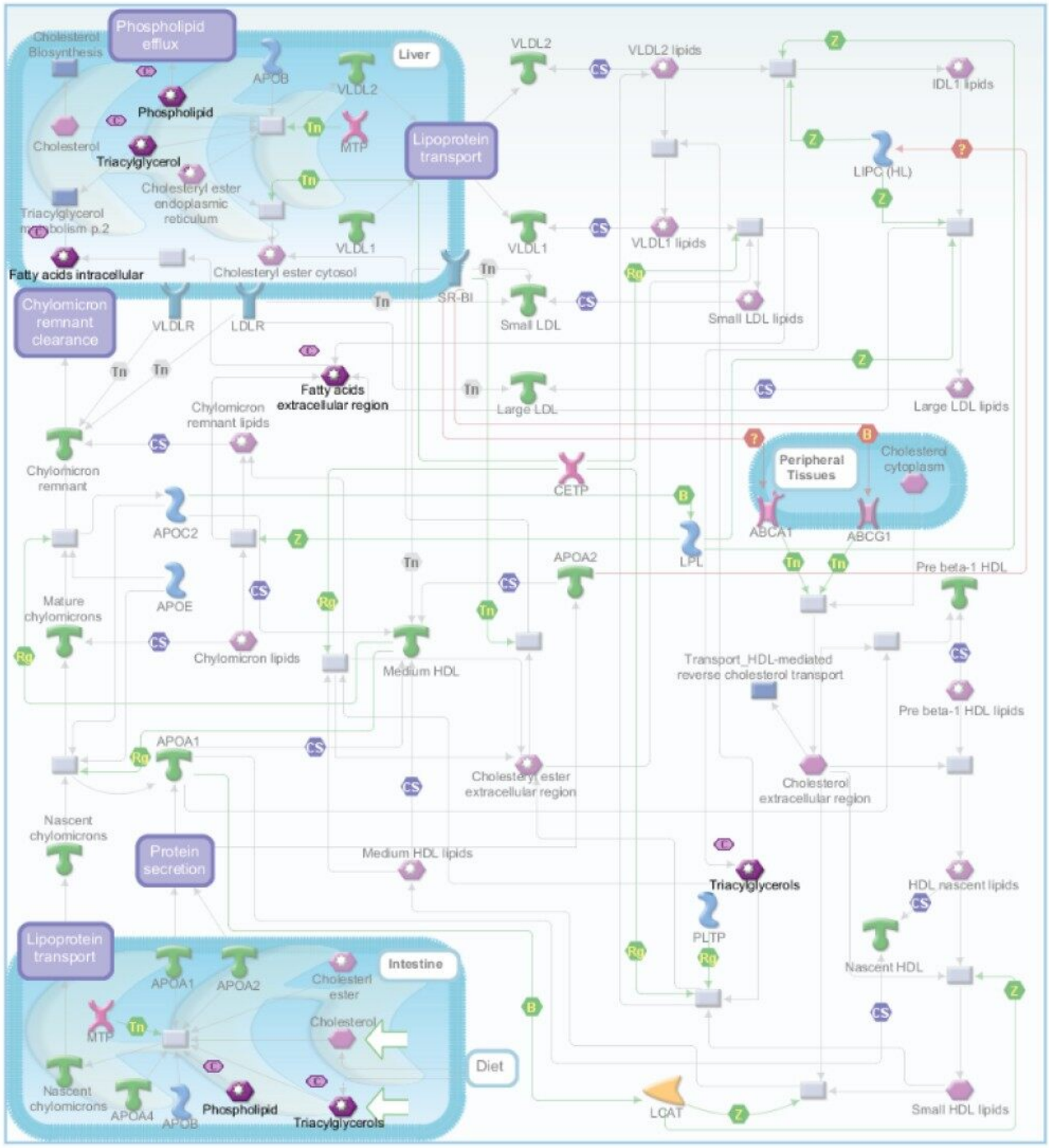

Figure 7. Lipoprotein metabolism pathway identified as being dysregulated for COPD diagnosed patients. A number of lipid components including phospholipids, triacylglycerols, and fatty acids involved in lipoprotein transportation are highlighted.

Conclusion

- A rapid, quantitative method (LipidQuan) was used to analyze the plasma from patients diagnosed with COPD and asthma in comparison to healthy controls.
- The analytical methodology, combined with multivariate statistics employing 447 lipid species as variables, differentiated sample cohorts by disease state.
- Lipids were quantified over 4-orders of dynamic range, allowing for sufficient limits of detection and quantification of endogenous lipids from human plasma.
- A number of lipid species were identified as being differentially regulated (FFAs, PCs, LPCs, SMs and Ceramides) between disease states. Data integration and pathway analysis revealed a number of disease-associated biological processes, including inflammation, oxidative, and immunity processes, were dysregulated in disease cohorts.

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