Biomarkers of Early Chronic Obstructive Pulmonary Disease (COPD) in Smokers and Former Smokers

- A comparison of two methods

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December, 2015

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Contents

1. Introduction

Chronic obstructive pulmonary disease (COPD) is an irreversible disease, diagnosed predominantly in smokers. COPD is the third leading cause of death worldwide (1,2). It is a heterogeneous inflammatory disease characterized by different phenotypes, such as airflow limitation that is not fully reversible, chronic sputum production (chronic bronchitis) and destruction of parenchymal lung tissue (emphysema) (2). Active smoking is the main risk factor for COPD. Far more than 15% of smokers get COPD: in fact, most develop some degree of pulmonary impairment (3). Smoking-related COPD is also associated with systemic manifestations such as weight loss, osteoporosis, depression, lung cancer, and cardiovascular disease (4). The number of inflammatory cells and mediators are increased in the lungs and can be detected in bronchoalveolar lavage, biopsies and blood (5). The pathophysiology of these systemic manifestations is unclear; however, several attempts both in cross-sectional and longitudinal studies have been performed, studying biomarkers closely associated with COPD phenotypes (6). One study, for example, found significant differences in biomarkers when comparing patients with COPD with former or non-smoking controls (7). Some biomarkers had large variability within the three month replication period. However, plasma fibrinogen was the most reproducible target and was elevated, along with plasma CRP, in COPD patients with exacerbations compared with COPD patients without exacerbations. There are recent strategies to classify structural changes in COPD using CT-imaging of morphological changes in pulmonary tissue tracts (8).

However, spirometry, forced expiratory volume in one second (FEV_1) is most widely used to evaluate the disease severity with increasing airflow limitations (2,9). FEV_1 correlates poorly to clinical symptoms in early stages of COPD, but performs slightly better in later stages of the disease (2). The ratio of FEV_1 to forced vital capacity (FVC) is measured with spirometry and is currently used to classify the disease state of COPD according to the Global initiative for Obstructive Lung Disease (GOLD) stages 1-4 (10). The most recent GOLD classification includes both symptoms and "Quality of Life" (QoL) metrics, in addition to lung function (11).

Smoking-related COPD is associated with acute exacerbations and is closely correlated to comorbidities, such as cardiovascular disease and lung cancer (2). The COPDGene study aims to define both genetic factors implicated in COPD, as well as to investigate structural changes and "Quality of Life" (QoL) questionnaires. The original cross-sectional study, which initiated in 2008, has been extended with several follow-up studies to detect changes during disease progression (12).

Over the last decade, several studies have attempted to identify new diagnostic biomarkers of COPD in blood, as well as to predict exacerbations and to classify disease states (13). However, there is still a lack of reliable biomarkers that optimize diagnostics and personalize the treatment of patients with COPD.

A panel of well-defined protein markers used in clinical diagnostics is analyzed at the Department of Clinical Chemistry at Skåne University Hospital in Malmö, Sweden and is compared with nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS) analysis at Centre of Excellence in Biological and Medical Mass Spectrometry (CEBMMS), Lund University. The aim of the study is to

compare the different assays to prepare future studies of identifying biomarkers of COPD in patient blood samples.

2. Methods

2a. Study Outline

This study is a close collaboration between Örestadskliniken (a primary health care clinic in Malmö), Skåne University Hospital in Malmö and Lund, and CEBMMS, at Lund University. The study was initiated early 2014 and has a five year duration. Two hundred smokers and ex-smokers with diagnosed COPD according to the GOLD stage 1-4 will be compared with a control group of 50 healthy never-smokers and 50 healthy smokers/ex-smokers (**Table 1**).

Exclusion criteria include presence of a chronic inflammatory disease, treatment with steroids, or any other immunomodulatory treatment that is unrelated to exacerbations of COPD. These conditions may otherwise interfere with the biomarker measurements in the study. Smokers with COPD must have at least 20 pack years in order to qualify for the GOLD stage groups.

Study groups by Pack years and flow limitation	**Pack years 1 pack year = 20 cigarettes a day in one year**	**Airflow limitation** FEV_1/FVC < 70%
Healthy never smokers n=50	0	No
Healthy smokers/ex-smokers n=25	< 20	No
At risk smokers/ex-smokers n=25	> 20	No
GOLD stage 1 smokers/ex-smokers n=75	> 20	$FEV_1 \geq 80\%$
GOLD stage 2 smokers/ex-smokers n=75	> 20	$50\% \leq FEV_1 < 80\%$
GOLD stage 3 smokers/ex-smokers n=25	> 20	$30\% \leq FEV_1 < 50\%$
GOLD stage 4 smokers/ex-smokers n=25	> 20	$FEV_1 < 30\%$

Table 1. The enrolled subjects will be divided in the following study groups. The total number of participants will be 300, including 50 healthy never smokers, 25 healthy smokers and ex-smokers, 25 at risk smokers and ex-smokers, 200 participants with GOLD stage 1-4 divided in 75 with GOLD stage 1 and 2 respectively (smokers and ex-smokers) and 25 with GOLD stage 3 and 4 respectively COPD patients (smokers and ex-smokers). All at-risk smokers and GOLD stage smokers have more than 20 pack years.

The participants will undergo health examination including heart and lung, blood pressure, and spirometry. They also fill out a QoL questionnaire regarding smoking habits, COPD symptoms, disease history and current medication. Blood samples are collected every six months, and are processed and stored as individual samples of plasma, buffy coat, red blood cells, serum and whole blood. Blood samples are collected in separate tubes (vacutainers) containing sodium heparin, sodium citrate or ethylenediaminetetraacetic acid (EDTA) as anticoagulants, or clot activator for

serum separation. Separate plasma tubes (heparin) are used to analyze a defined list of biomarkers at the Clinical Chemistry Laboratory at Skåne University Hospital in Malmö. Additional blood sample collection is performed when subjects experience an exacerbation. This study is approved by the Regional Ethical Review Board in Lund (Approval number: DNR 2013/480).

All data are stored in a clinical database, utilizing Research Electronic Data Capture (REDCap) as a data capture tool (14) and the blood samples are subsequently analyzed by LC-MS/MS at CEBMMS, Lund University.

2b. Clinical Samples and Biobank

The biobanking workflow has previously been developed at CEBMMS for rigorous standardization for large scale biobanking (15-18). The blood samples are collected at the primary health care clinic and transported by a courier to the biobank at CEBMMS for aliquoting and storage. Four vacutainers are centrifuged at 2000 g for 10 min in order to separate blood into three fractions: plasma, buffy coat and red blood cells. An automated robotic system (HAMILTON robot MicroLab Starlet, Hamilton, Bonaduz AG, Switzerland) aliquots 70 µL of sample into 192 vials per subject into the 384-well plate. Hence, aliquots from two participants can fit in the same 384-well plate. The vials arriving from the clinic have a 1-D barcode and are matched with another 1-D barcode on the 384-well plate. Subsequently, the vials are sealed with aluminum foil and punched individually. All racks are stored at -80 ^{o}C in a local biobank freezer (LICONIC freezer STT1k5 ULT, Liconic AG, Mauren,

Liechtenstein). This procedure, from sample collection to storage in the biobank, takes less than two hours, which ensures safe handling of the samples, as well as high sample quality. Producing multiple aliquots from the same sample avoids the freezing-and-thawing procedure, which can otherwise compromise sample quality. Each subject is assigned a unique identifier (Study ID) allowing all samples and data to be handled anonymously. The subject study identity can only be deciphered by clinicians at Örestadskliniken. For analysis, the aliquot vials can be retrieved via electronic command through the computerized biobanking system.

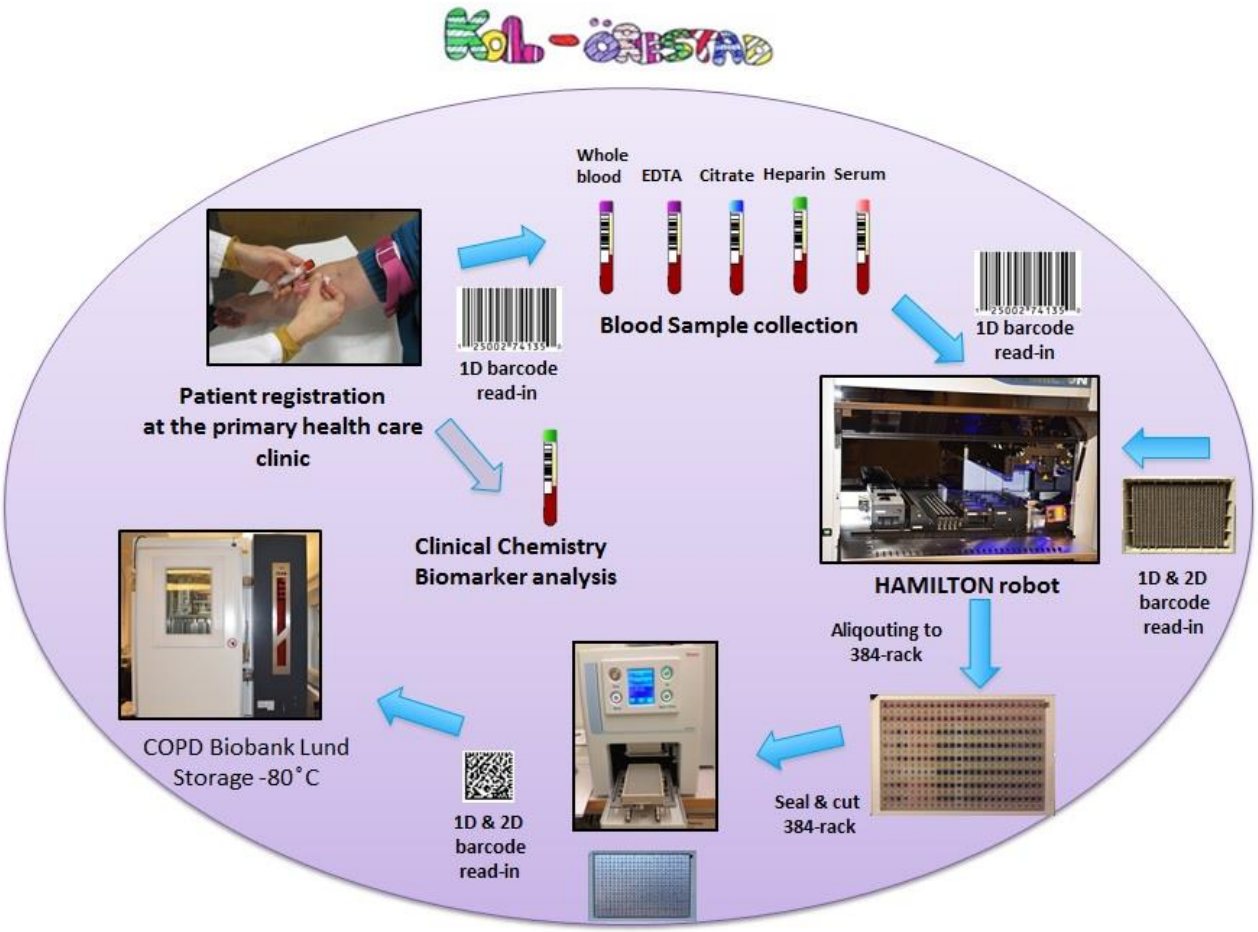

Figure 1. Illustration of the flow of clinical samples: bar-coding, sorting, sealing and storage in the biobank freezer. The cycle time is less than two hours.

2c. Clinical Database

The data acquired are stored in a database, KOL-Örestad database, including clinical data of the participants: blood pressure, spirometry, and a QoL questionnaire detailing smoking habits, COPD symptoms and disease history. By use of the barcode or study ID, all clinical demographics, type of blood samples, and analyzed output data stored in the database, can be traced from each participant.

REDCap from Vanderbilt University, TN, USA was utilized as a database platform, because it provides a flexible and easily programmable interface especially adapted for clinical studies (14). REDCap has user-specified input modules for a specific cluster of variables, e.g. modules on: QoL, blood sampling, spirometry, without restriction in time and numbers. User allowance can be adjusted for different users, e.g. clinicians and researchers. This preserves participant integrity by maximizing the flexibility of the platform. All participant data that is handled outside the health care sector is encrypted and is handled anonymously.

REDCap also functions as a planning instrument for clinicians, facilitating the planning of the number of patient follow ups each participant has completed. This gives the involved clinicians and researchers an excellent overview of all participants and their level of completion in the study.

Health Information System (HIS) provides the foundation for decision-making and is comprised of four key functions: data generation, compilation, analysis and synthesis, and communication and use. Data from the health care sector is collected within HIS, as well as analyzed and controlled for quality, relevance and timeliness (19). The flow of sample and data in the study can be viewed in **Figure 2**.

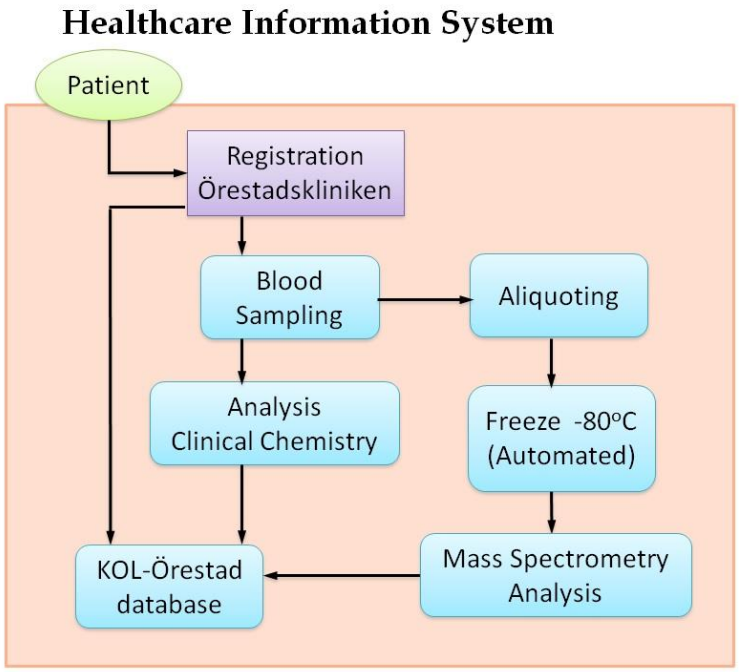

Figure 2. A demonstration of the workflow with Health Information System (HIS).The patient is registered at Örestadskliniken, the primary health care clinic. Blood samples are sent to Clinical Chemistry at the Skåne University Hospital in Malmö and to CEBMMS for analysis and storage in the biobank. These data, as well as clinical data from Örestadskliniken, are collected within the KOL-Örestad database.

2d. Data Analysis

In the present study, 20 subjects were selected, ten healthy (non-smokers, passive smokers) persons were compared with ten COPD patients with GOLD-stage 1-2. Samples were prepared according to the protocol (**Attachment 1**).

EDTA plasma samples diluted with 50 mM ammonium bicarbonate 1:10 (v/v) were denatured with 5% (w/v) sodium deoxycholate (SDC) at 80 °C for 10 min, reduced with 10 mM dithiothreitol (DTT) at 60 °C for 60 min and alkylated with 50 mM iodoacetamide (IAA) at room temperature for 30 min in darkness. Chicken lysozyme (Sigma Cat. No L6876, UniProt # P00698) was used as internal standard and was

added to each sample before reduction with DTT. After SDC concentration was reduced to 0.5% with 50 mM ammonium bicarbonate, samples were digested with trypsin (trypsin:protein ratio 1:100 w/w) for 18 hours at 37 °C. Formic acid was added to stop the digestion and precipitate SDC. Samples were centrifuged for 15 min at 16000 g and 70 µL of supernatant was transferred into a new Eppendorf tube (**Figure 3**). Final dilution of plasma after digestion was 1:200 (40 µg protein/100 µL).

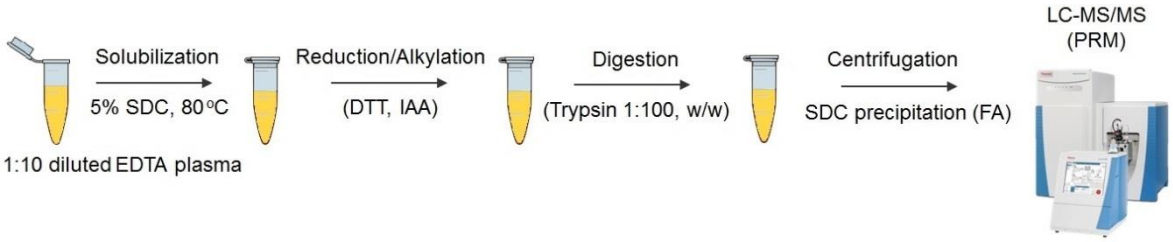

Figure 3. Samples were prepared for nLC-MS/MS data analysis by solubilization, reduction/alkylation with DTT and IAA, digestion and SDC precipitation.

Then, the samples were spiked with a mixture of synthetic heavy labeled peptides (Absolute QUAntification, AQUA, Thermo Scientific); and crude standards (JPT Peptide Technologies GmbH, Germany) for quantitative nLC-MS/MS analysis. Peptide mixtures were separated using an nLC II system (Easy nLC, Thermo Fisher Scientific) with a 300 nL/min flow rate. The peptides produced by digestion were first loaded onto a trap column (Acclaim® PepMap 100 pre-column, 75 µm x 2 cm, C18, 3 µm, 100 Å, Thermo Scientific, San José, CA) and then separated on an analytical column (EASY-Spray column, 50cm x 75µm ID, PepMap RSLC C18, 2µm, 100Å, Thermo Scientific, San José, CA). Solvent A, consisting of 0.1% formic acid in

water, and Solvent B, consisting of 0.1% formic acid in acetonitrile, were used as a mobile phase. The peptides were eluted with a 90-min linear gradient from 5% B to 40% B, followed by a 5-min linear gradient to 90% B and a column wash at 90% B for 10 min. Analyses of EDTA plasma peptides were scheduled in parallel reaction monitoring (PRM) and acquired on a Q-Exactive Plus mass spectrometer (Thermo Scientific, San José, CA). The peptides were measured with a scheduled inclusion list and were measured in duplicate. The conformation of identities was carried out based on prior data dependent analysis (DDA) of pooled plasma samples (from participant #1-10 and #11-20) and library obtained from the in Proteome Discoverer database search (v 1.4, Thermo Scientific, San José, CA). Fragment ion patterns were compared with the reference spectra acquired in the DDA and an isolation list with 5-min retention time window for each of the peptide was created. The following parameters were utilized: 17,500 resolution, Automatic Gain Control (AGC) target 1×10^5, 50 ms maximum injection time, and 1.6 m/z isolation window. Ion activation/dissociation was performed by HCD at a normalized collision energy of 35%. The list of proteins and their corresponding peptides analyzed by PRM (isolation list) with a ratio (heavy to light peptide) was based on the measurements for pooled plasma of samples #1-10 (**Attachment 2**).

Quantitation was performed with heavy and light peptides of AQUA quality with one peptide per protein for apolipoprotein A1 (apoA1), apolipoprotein B (apoB) and C-reactive protein (CRP) and crude heavy peptide standards for fibrinogen alpha (fibα) and fibrinogen beta (fibβ). Calibration solutions of light peptides were prepared in a Solvent A and calibration solutions of heavy peptides were prepared in

pooled plasma samples (from participant #1-10 and #11-20). A mixture of heavy peptides was added to each sample. Optimal ratio of light to heavy peptides for quantification was determined based on the calibration curves for each peptide.

To calculate the concentration of the endogenous sample, the slope of the calibration curve was utilized. In the last step of the calculation, the concentration was multiplied by 200, because the sample was diluted 1:200 during sample preparation.

The protein concentration from the nLC-MS/MS from EDTA plasma samples were compared with concentrations measured at Clinical Chemistry from the same participant blood samples from heparin plasma samples. Heparin tubes are standardly used at Clinical Chemistry, but for mass spectrometry, which is a method of higher sensitivity, EDTA tubes are preferred because it provides a more robust protein and peptide sample for biomarker discovery and clinical analysis (20).

Student T-test was utilized in order to determine if there were any significant differences between the healthy participants and the participants with COPD GOLD stage 1-2. The test was two-tailed and two-sample equal variance (homoscedastic).

The methods (nLC-MS/MS and clinical chemistry assay) were compared calculating the significance of correlation coefficients. The following equation was utilized:

$$t = \frac{\mathbf{r}}{\sqrt{[(1 - \boldsymbol{r^2})/(\mathrm{N} - 2)]}}$$

Where t = the significance of the correlation coefficient, r = correlation coefficient, r^2 = correlation of determination, and N = subject number in the comparison groups.

The square of the correlation coefficient is calculated in Excel (Microsoft 2010) when a scatter plot between the methods is created.

3. Results

The PRM assay was optimized for the list of proteins on the nLC-MS/MS Q-Exactive plus (**Attachment 2**). Of the 24 proteins measured, there were heavy peptides available for 15 of the proteins (alpha-1-acid glycoprotein, alpha-2-macroglobulin, apoA1, apoB, apolipoprotein C3, ceruloplasmin, complement C3, complement C4, clusterin, CRP, fibα, fibβ, haptoglobin, hemopexin, and plasminogen). An overview of the nLC-MS/MS run in PRM of all the samples in the study can be viewed in **Figure 4**, as well as an example of a biomarker across the samples.

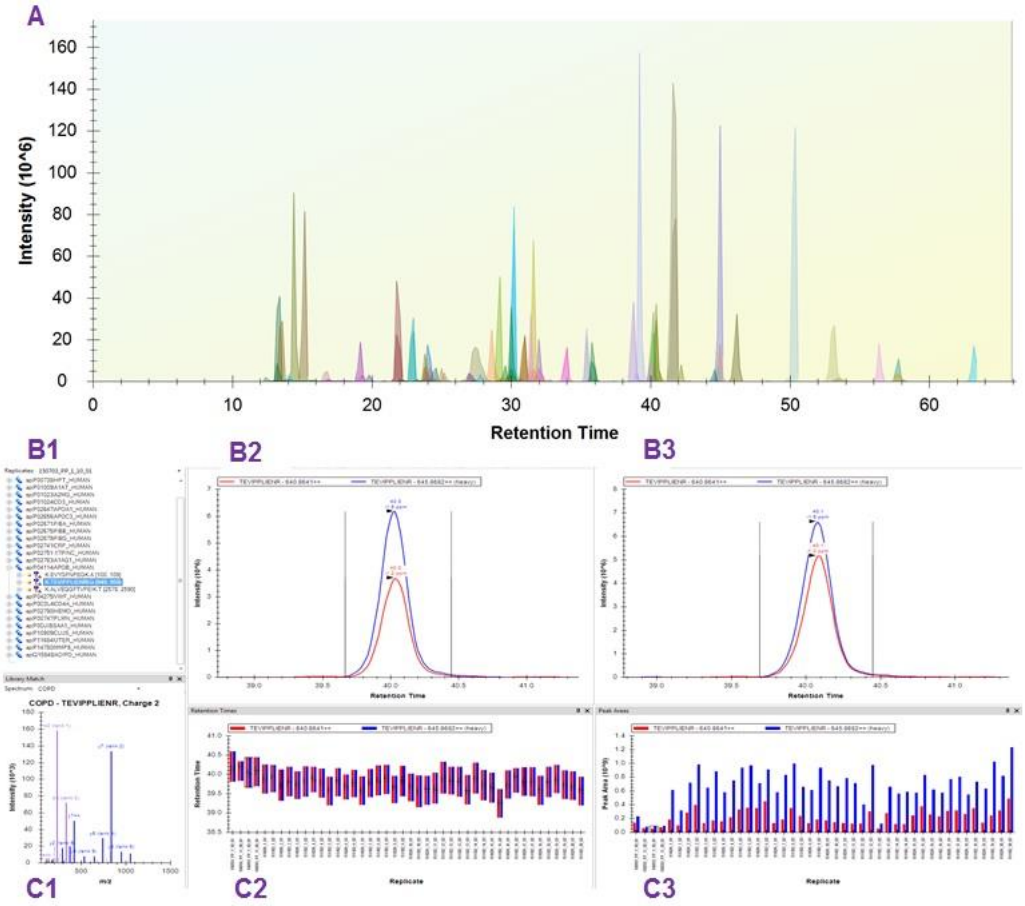

Figure 4. A) Demonstrates that there was adequate separation of proteins across the samples analyzed. Each peak represents a different protein. B1) A part of the protein and peptide list of the proteins analyzed by nLC-MS/MS in Skyline. B2 and B3) The two peaks are from two separate

samples, where the blue peak corresponds to the heavy labeled peptide and the red corresponds to the endogenous peptide. C1)MS/MS data from the endogenous peptide. C2) The retention time of that same peptide across all samples (blue = heavy labeled peptide, red = endogenous). C3) The intensities of the peptide across all samples.

Calibration curves were constructed for the peptides corresponding to the 15 proteins and the samples were measured in duplicate. The present study was focused on three classical proteins that also are determined in many standard clinical chemistry assays, thus, enabling comparison with the developed nLC-MS/MS parameters. Calibration curves were constructed and quantification of endogenous peptides was performed for apoA1, apoB and CRP. Fibα and fibβ were also quantified with nLC-MS/MS, because they have been observed as interesting findings in other COPD studies (5, 7). Calibration curves for each relevant peptide standards and figures comparing quantification data between the two subject groups are presented (**Figure 5-8**). The calibration curves for apoA1, apoB and CRP were constructed with heavy and light calibration standards of AQUA quality. To determine endogenous concentration of fibrinogen heavy crude peptides were used. Tables of the nLC-MS/MS and clinical chemistry assays for samples 1-20 can be viewed in **attachment 3 (Suppl. Table 1-3)**. Additionally, the nLC-MS/MS for fibα and fibβ for samples 1-20 is shown in **attachment 4 (Suppl. Table 4 and 5)**.

Apolipoprotein A1

The equation and correlation of determination (r^2) from the calibration curve of apoA1 was y = 3526.9x – 0.033 and r^2 = 0.997. There were 6 measuring points on the calibration curve (range 0.0000057 g/L – 0.0002326 g/L), and the calibration solutions were only measured once. The measurements from the nLC-MS/MS had an average standard deviation (SD) of 0.43 and coefficient of variation in percent (CV) of 4.52%. There was no significant difference between the groups within each method (nLC-MS/MS, p = 0.612, and Clinical Chemistry, p = 0.464).

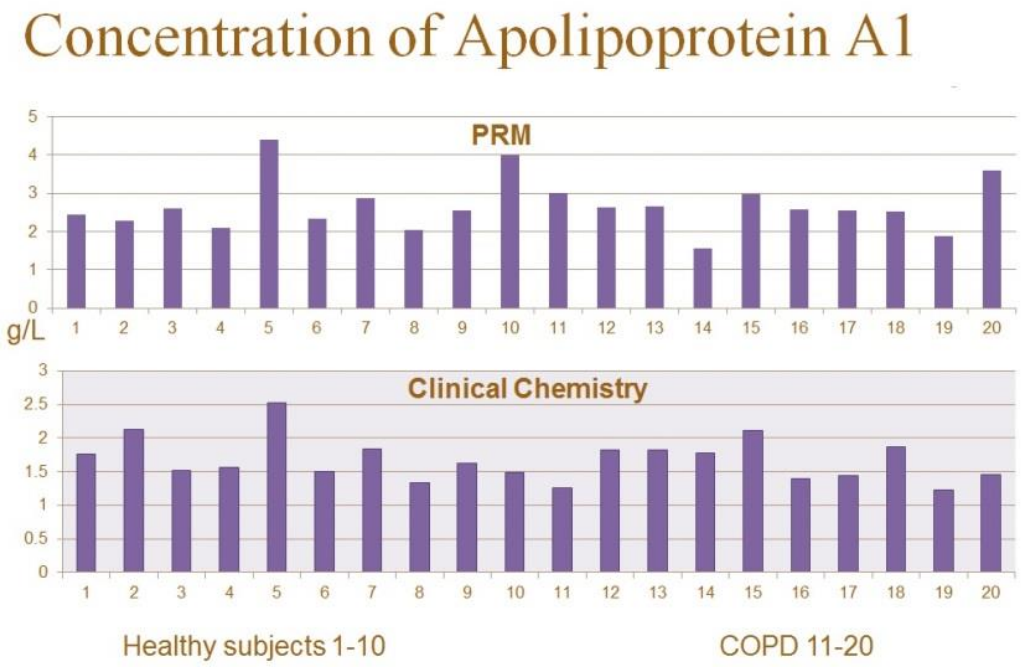

Figure 5. The above graph represents the quantity of apoA1 measured with nLC-MS/MS in PRM mode and below is the quantity of apoA1 measured with standard clinical chemistry assay.

The obtained values from the nLC-MS/MS and the standard clinical assay were plotted against each other and analyzed using a scatter chart (Excel 2010), y = 0.1645x + 1.2341 and r^2 = 0.1163. There was no significant correlation between the two assays (p = 0.312).

Apolipoprotein B

The equation and r^2 value from the calibration curve of apoB were $y = 4271.6x - 1.5639$ and $r^2 = 0.991$. There were 9 measuring points on the calibration curve (range 0.0000402 g/L – 0.0103000 g/L) and measured in duplicate, CV <25%. The measurements from the nLC-MS/MS analysis had an average SD of 0.01 and CV of 1.85%. There was no significant difference between the groups within each method (nLC-MS/MS, $p = 0.370$, and Clinical Chemistry, $p = 0.218$).

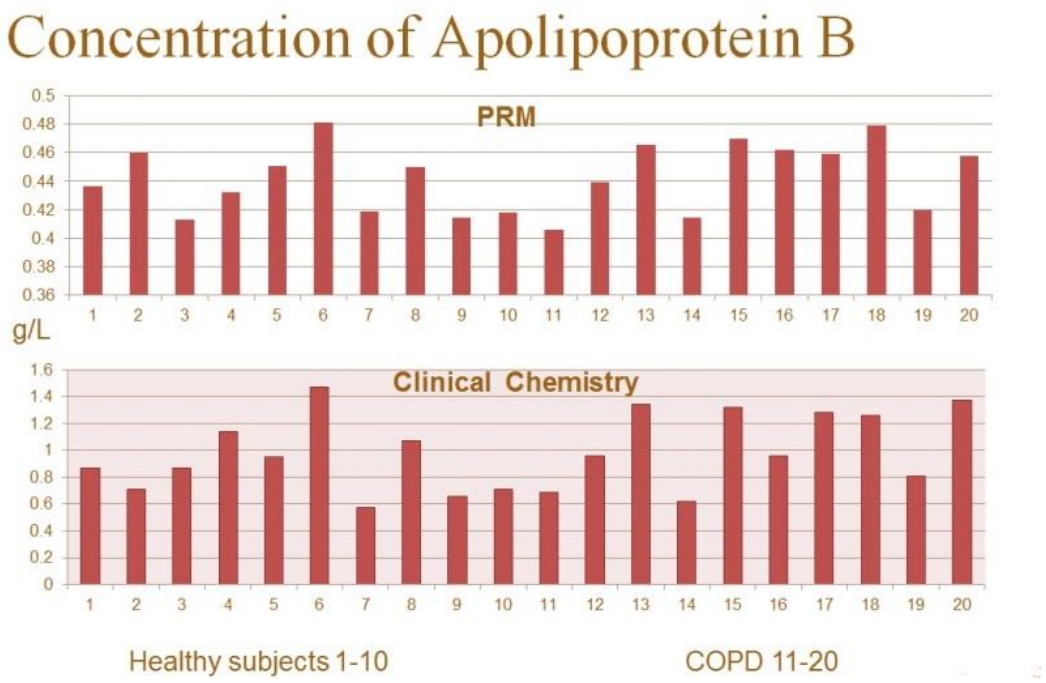

Figure 6. The above graph represents the quantity of apoA1 measured with nLC-MS/MS in PRM mode and below is the quantity measured with standard clinical chemistry assay.

The obtained values from nLC-MS/MS and the standard clinical assay were plotted against each other: $y = 9.5448x - 3.2393$ and $r^2 = 0.6594$. The correlation between methods was significant ($p = 0.001$).

C-reactive protein

The equation and r^2 value from the calibration curve of CRP were

$y = 3951.4x - 0.1324$ and $r^2 = 0.996$. There were 8 measuring points on the calibration curve (range 0.0000449 g/L – 0.0115000 g/L) and measured in duplicate, CV <76%. The measurements from the nLC-MS/MS analysis had an average SD of 0.03 and CV of 41.41%. There was no significant difference between the groups within each method (nLC-MS/MS, $p = 0.090$, and Clinical Chemistry, $p = 0.192$).

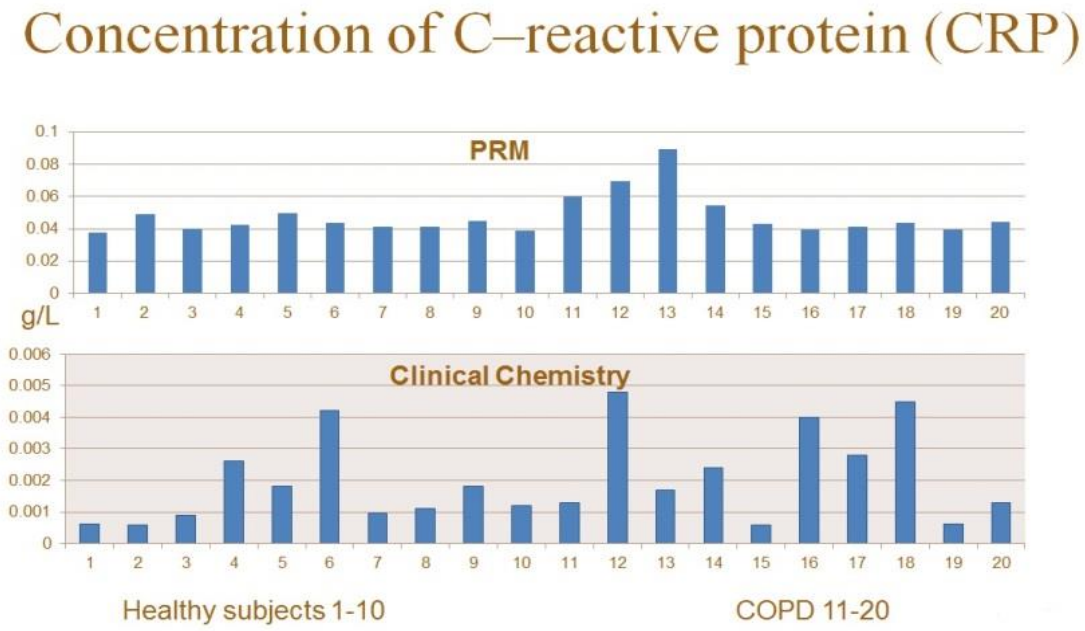

Figure 7. The above graph represents the quantity of apoA1 measured with nLC-MS/MS in PRM mode and below is the quantity of CRP measured with standard clinical chemistry assay.

The obtained values from nLC-MS/MS and the standard clinical assay were plotted against each other: $y = 0.0198x + 0.001$ and $r^2 = 0.0322$. The correlation between methods was not significant ($p = 0.446$).

Fibrinogen α & β

The concentrations of fibrinogen, which was only measured with nLC-MS/MS, is within the reference range for clinical blood samples, 2.0 -4.0 g/L (21). However, the reference value has no distinction between fibα & fibβ chains. For fibα, the equation and r^2 value from the calibration curve were y = 1697.1x + 0.2647 and r^2 = 0.998. There were 8 measuring points on the calibration curve (range 0.0000468 g/L – 0.0237475 g/L) and the measurements were in duplicate, CV <29%. For fibβ, the results were y = 3245.5x – 3.3304 and r^2 = 0.9925 with 6 measuring points on the calibration curve (range 0.0004370 g/L – 0.0139850 g/L) measured in duplicate, CV <61%. The measurements from the nLC-MS/MS of fibα had an average SD of 0.20 and CV of 4.32% and fibβ had 0.37 and 17.03% respectively. There was no significant difference between the groups within each chain (fibα p = 0.081 and fibβ p = 0.144).

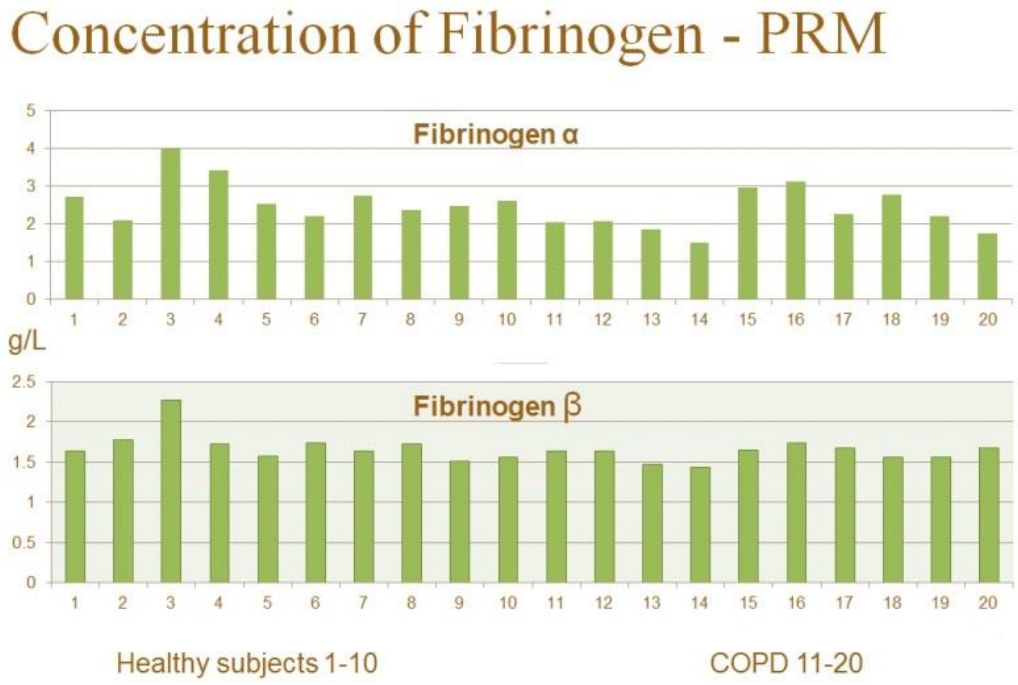

Figure 8. The above graph contains the concentration of fibα and the graph below contains the concentration of fibβ. Both were measured by nLC-MS/MS in PRM mode.

The nLC-MS/MS measurements of fibα and fibβ were plotted against each other, which resulted in $y = 0.1916x + 1.1839$ and $r^2 = 0.4435$. The correlation between the fibrinogen chains was significant ($p = 0.025$).

4. Discussion

In this study, ten COPD patients with GOLD-stage 1-2 were compared to ten healthy subjects in order to assess COPD in its early stages; however, there were no significant differences observed between the two study groups using nLC-MS/MS quantitation or standard clinical chemistry assays. When comparing the two assay measurements, only apoB had a significant correlation between the methods. The concentration difference between the two methods could be explained by the samples measured being collected in different types of tubes: EDTA tubes for the nLC-MS/MS quantitation and heparin tubes for the clinical chemistry assay. Another potential reason for the difference between the two methods is that the assays utilize two completely different approaches, mass spectrometry in the nLC-MS/MS assay and antibody-based turbidimetry in the clinical chemistry assay.

Fibrinogen and CRP were of particular interest to compare between healthy and COPD subjects, due to the known pathological increase in patients with COPD (22). There is, however, a gray area between healthy persons and individuals diagnosed with COPD due to the lungs natural ageing process and decline in FEV_1/FVC (2, 23). The heterogeneity between sample groups would be ameliorated by comparing

healthy participants with patients with GOLD stage 3-4 or healthy participants with patients with COPD experiencing an exacerbation. This could minimize the risk of having healthy subjects misdiagnosed with early COPD in the COPD subject group, and vice versa. The differences in quantification results between the methods utilized may be due to the method not being optimized for each protein biomarker of interest. For example, the signal obtained for CRP endogenous peptide measured with the nLC-MS/MS method had a very low intensity and a poor fragmentation pattern. This can be improved if a lower concentration of the heavy standard for CRP is added to the plasma prior to the nLC-MS/MS run, because it would improve the ratio of heavy to light peptide. The endogenous concentration of CRP in plasma is in the concentration range of mg/L; thus, other methods, such as pre-fractionation, may aid in increasing the concentration of CRP and potentially improve nLC-MS/MS quantification of CRP (21, 24).

All clinical samples were measured in duplicate. Although data was consistent in general, some CV values were higher than 25%. The data quality of the study would be improved with an increased number of runs and by using the same kind of tube for the blood sample collection (e.g. EDTA), as well as larger subject groups.

5. Conclusion

There were no significant differences between the subject groups with COPD GOLD stage 1-2 and the healthy control in the protein levels analyzed. When comparing the two methods, nLC-MS/MS and clinical chemistry assay, the only protein with a significant correlation was apoB (p = 0.001). However, fibrinogen was not measured with the clinical chemistry assay, so the coefficient of correlation could not be calculated. The correlation between the concentration levels of the fibrinogen chains (fibα and fibβ) measured with nLC-MS/MS was significant (p = 0.025).

The findings in this study do not collectively have a statistical significance; however, the study provides good insight in how to optimize the current nLC-MS/MS method and sample preparation for the continuation of the study.

The study is set up to follow the disease progression longitudinally for each patient and relate novel identified disease biomarkers that are significantly up or down regulated due to disease status from measured clinical parameters. The study design may enable the discovery of new biomarkers that define early changes of COPD, distinct from markers of closely related diseases, e.g. cardiovascular disease and lung cancer. Such panels of novel biomarkers may be able to contribute to an increased understanding of these diseases.

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Attachment 1

Protocol for digestion of COPD EDTA plasma sample with SDC; DTT, IAA, Trypsin

1) Place 10 µL of 5% (w/v) SDC in 50 mM AmBic in 1.5 mL Eppendorf tube

2) Add 5 µL plasma diluted 1:10 with 50 mM AmBic; 0.5 µL plasma = 40 µg protein

3) Add 4 µL chicken lysozyme (0.01 µg/µL)

4) Incubate in Thermoblock for 10 min at 80 °C

5) Add 5 µL 50 mM DTT in 50 mM AmBic (final concentration 10.4 mM DTT)

6) Incubate in Thermoblock for 60 min at 60 °C

7) Add 8 µL 200 mM IAA in 50 mM AmBic (final concentration 50 mM IAA)

8) Incubate in Thermoblock for 30 min at room temperature in dark (foil)

9) Add 61 µL of 50 mM AmBic to reduce concentration of SDC to 0.5% before adding trypsin

10) Add 0.4 µg of trypsin (trypsin:protein 1:100 w/w) (20 µg/100 µL stock in resuspension buffer, stored at -20 °C)

11) Incubate in Thermoblock overnight (ca 18 h) at 37 °C

12) Add 5 µL of 10% formic acid to stop the digestion and precipitate SDC

13) Centrifugation for 15 min at 16000 g

14) Transfer 70 µL into a new Eppendorf tube

15) Final dilution of plasma after digestion is 1:200 (40 µg protein/100 µL)

Attachment 2

List of proteins and their corresponding peptides analyzed with nLC-MS/MS in PRM mode. Ratio light to heavy as measured for pooled plasma samples #1-10.

Protein Name	Modified Sequence	Peptide RT	Ratio L/H	Precursor Charge	Precursor Mz
sp|P00450|CERU_HUMAN	EYTDASFTNR	23.65	0.5145	2	602.267467
sp|P00450|CERU_HUMAN	EYTDASFTNR[+10]	23.65	0.5145	2	607.271601
sp|P00450|CERU_HUMAN	GAYPLSIEPIGVR	45.15	#N/A	2	686.385176
sp|P00450|CERU_HUMAN	QYTDSTFR	19.18	#N/A	2	509.235438
sp|P00698|LYSC_CHICK	GYSLGNWVC[+57]AAK	42.45	#N/A	2	663.318979
sp|P00698|LYSC_CHICK	NTDGSTDYGILQINSR	39.82	#N/A	2	877.421204
sp|P00698|LYSC_CHICK	NLC[+57]NIPC[+57]SALLSSDITASVNC[+57]AK	51.81	#N/A	3	836.734553
sp|P00738|HPT_HUMAN	TEGDGVYTLNDK	24.74	#N/A	2	656.306789
sp|P00738|HPT_HUMAN	TEGDGVYTLNNEK	23.98	#N/A	2	720.336078
sp|P00738|HPT_HUMAN	QLVEIEK	22.49	#N/A	2	429.750193
sp|P00738|HPT_HUMAN	VGYVSGWGR	30.13	2.5445	2	490.751058
sp|P00738|HPT_HUMAN	VGYVSGWGR[+10]	30.13	2.5445	2	495.755193
sp|P00738|HPT_HUMAN	YVMLPVADQDQC[+57]IR	40.76	#N/A	2	854.413282
sp|P01009|A1AT_HUMAN	ITPNLAEFAFSLYR	63.85	#N/A	2	821.435398
sp|P01009|A1AT_HUMAN	QINDYVEK	19.03	#N/A	2	504.753464
sp|P01009|A1AT_HUMAN	SVLGQLGITK	39.29	#N/A	2	508.310949
sp|P01023|A2MG_HUMAN	NEDSLVFVQTDK	35.3	1.644	2	697.843538
sp|P01023|A2MG_HUMAN	NEDSLVFVQTDK[+8]	35.3	1.644	2	701.850638
sp|P01023|A2MG_HUMAN	QGIPFFGQVR	46.54	#N/A	2	574.81419
sp|P01023|A2MG_HUMAN	LLIYAVLPTGDVIGDSAK	57.89	#N/A	2	923.022034
sp|P01024|CO3_HUMAN	LSINTHPSQKPLSITVR	30.58	#N/A	4	473.52445
sp|P01024|CO3_HUMAN	LVAYYTLIGASGQR	44.73	#N/A	2	756.414465
sp|P01024|CO3_HUMAN	TGLQEVEVK	23.01	1.2337	2	501.776939
sp|P01024|CO3_HUMAN	TGLQEVEVK[+8]	23.01	1.2337	2	505.784038
sp|P01024|CO3_HUMAN	SGSDEVQVGQQR	13.43	#N/A	2	645.307654
sp|P02647|APOA1_HUMAN	DYVSQFEGSALGK	44.72	#N/A	2	700.838256
sp|P02647|APOA1_HUMAN	LLDNWDSVTSTFSK	50.3	#N/A	2	806.896302
sp|P02647|APOA1_HUMAN	ATEHLSTLSEK	13.92	9.0621	3	405.878703
sp|P02647|APOA1_HUMAN	ATEHLSTLSEK[+8]	13.92	9.0621	3	408.550103
sp|P02656|APOC3_HUMAN	DALSSVQESQVAQQAR	30.55	1.2149	3	572.955223
sp|P02656|APOC3_HUMAN	DALSSVQESQVAQQAR[+10]	30.55	1.2149	3	576.291313
sp|P02656|APOC3_HUMAN	GWVTDGFSSLK	44.63	#N/A	2	598.800945
sp|P02671|FIBA_HUMAN	NSLFEYQK	29.23	#N/A	2	514.756006
sp|P02671|FIBA_HUMAN	GGSTSYGTGSETESPR	14.3	#N/A	2	786.842255
sp|P02671|FIBA_HUMAN	GSESGIFTNTK	21.98	0.4022	2	570.78021
sp|P02671|FIBA_HUMAN	GSESGIFTNTK[+8]	21.98	0.4022	2	574.787309
sp|P02675|FIBB_HUMAN	DNENVVNEYSSELEK	40.09	0.6886	2	884.897228

sp | P02675 | FIBB_HUMAN	DNENVVNEYSSELEK[+8]	40.09	0.6886	2	888.904327
sp | P02675 | FIBB_HUMAN	TPC[+57]TVSC[+57]NIPVVSGK	31.54	#N/A	2	809.900008
sp | P02675 | FIBB_HUMAN	EDGGGWWYNR	38.74	#N/A	2	620.262519
sp | P02679 | FIBG_HUMAN	FGSYC[+57]PTTC[+57]GIADFLSTYQTK	58.01	#N/A	3	806.364454
sp | P02679 | FIBG_HUMAN	LDGSVDFK	24.36	#N/A	2	440.724175
sp | P02679 | FIBG_HUMAN	IIPFNR	27.51	#N/A	2	380.229231
sp | P02741 | CRP_HUMAN	ESDTSYVSLK	25.13	0.0512	2	564.774593
sp | P02741 | CRP_HUMAN	ESDTSYVSLK[+8]	25.13	0.0512	2	568.781693
sp | P02751-17 | FINC_HUMAN	YQC[+57]YC[+57]YGR	19.93	#N/A	2	585.228776
sp | P02751-17 | FINC_HUMAN	AQITGYR	14.17	#N/A	2	404.719227
sp | P02751-17 | FINC_HUMAN	VPGTSTSATLTGLTR	32	#N/A	2	731.399012
sp | P02763 | A1AG1_HUMAN	TEDTIFLR	34.01	#N/A	2	497.763831
sp | P02763 | A1AG1_HUMAN	EQLGEFYEALDC[+57]LR	56.77	#N/A	2	871.906344
sp | P02763 | A1AG1_HUMAN	SDVVYTDWKK	24.43	864.4719	3	414.211794
sp | P02763 | A1AG1_HUMAN	SDVVYTDWKK[+8]	24.43	864.4719	3	416.883193
sp | P04114 | APOB_HUMAN	EVYGFNPEGK	29.52	#N/A	2	570.272021
sp | P04114 | APOB_HUMAN	TEVIPPLIENR	40.14	0.6034	2	640.864077
sp | P04114 | APOB_HUMAN	TEVIPPLIENR[+10]	40.14	0.6034	2	645.868211
sp | P04114 | APOB_HUMAN	ALVEQGFTVPEIK	44.91	#N/A	2	715.898117
sp | P04275 | VWF_HUMAN	TATLC[+57]PQSC[+57]EER	17.72	#N/A	2	726.316308
sp | P0C0L4 | CO4A_HUMAN	YIYGKPVQGVAYVR	31.92	#N/A	3	538.299714
sp | P0C0L4 | CO4A_HUMAN	VGDTLNLNLR	35.89	1.2031	2	557.814387
sp | P0C0L4 | CO4A_HUMAN	VGDTLNLNLR[+10]	35.89	1.2031	2	562.818521
sp | P0C0L4 | CO4A_HUMAN	LQETSNWLLSQQQADGSFQDPC[+57]PVLDR	53.14	#N/A	3	1044.827885
sp | P0C0L4 | CO4A_HUMAN	ANSFLGEK	19.98	#N/A	2	433.224342
sp | P02790 | HEMO_HUMAN	ELISER	14.99	#N/A	2	373.705785
sp | P02790 | HEMO_HUMAN	NFPSPVDAAFR	41.61	0.5222	2	610.806562
sp | P02790 | HEMO_HUMAN	NFPSPVDAAFR[+10]	41.61	0.5222	2	615.810696
sp | P02790 | HEMO_HUMAN	LLQDEFPGIPSPLDAAVEC[+57]HR	53.48	#N/A	3	788.726603
sp | P00747 | PLMN_HUMAN	NLDENYC[+57]R	16.57	#N/A	2	542.22983
sp | P00747 | PLMN_HUMAN	ATTVTGTPC[+57]QDWAAQEPHR	28.01	#N/A	3	709.328843
sp | P00747 | PLMN_HUMAN	LFLEPTR	31.39	0.0983	2	438.252903
sp | P00747 | PLMN_HUMAN	LFLEPTR[+10]	31.39	0.0983	2	443.257037
sp | P0DJI8 | SAA1_HUMAN	FFGHGAEDSLADQAANEWGR	42.15	#N/A	3	726.659358
sp | P10909 | CLUS_HUMAN	EILSVDC[+57]STNNPSQAK	26.84	#N/A	2	881.91744
sp | P10909 | CLUS_HUMAN	ELDESLQVAER	29.78	0.1601	2	644.822606
sp | P10909 | CLUS_HUMAN	ELDESLQVAER[+10]	29.78	0.1601	2	649.82674
sp | P10909 | CLUS_HUMAN	VTTVASHTSDSDVPSGVTEVVVK	32.09	#N/A	3	772.063938
sp | P11684 | UTER_HUMAN	KLVDTLPQKPR	36.77	#N/A	3	432.266233
sp | P14780 | MMP9_HUMAN	AFALWSAVTPLTFTR	54.32	#N/A	2	840.95904
sp | Q15848 | ADIPO_HUMAN	IFYNQQNHYDGSTGK	16.85	#N/A	3	591.272663

Attachment 3.

Supplementary tables from the nLC-MS/MS quantification and standard clinical chemistry assay for respective proteins (ApoA1, ApoB and CRP). Subjects #1-10 are healthy and subjects #11-20 have COPD GOLD stage 1-2.

Subject	nLC-MS/MS g/L	Clin Chem g/L	AVG Ratio	SD	CV %
1	2.43	1.76	8.53	N/A	N/A
2	2.27	2.13	7.96	0.29	3.65
3	2.59	1.52	9.10	0.37	4.06
4	2.09	1.56	7.33	0.70	9.46
5	4.39	2.53	15.46	0.39	2.52
6	2.33	1.51	8.18	0.17	2.11
7	2.86	1.84	10.04	0.19	1.91
8	2.05	1.33	7.19	0.01	0.14
9	2.54	1.63	8.92	0.49	5.53
10	3.99	1.48	14.03	0.28	2.02
11	3.00	1.26	10.55	0.29	2.77
12	2.63	1.82	9.25	2.04	22.08
13	2.64	1.83	9.28	0.14	1.51
14	1.56	1.77	5.46	0.11	1.94
15	2.96	2.12	10.41	0.33	3.20
16	2.57	1.39	9.02	0.18	2.00
17	2.55	1.44	8.94	0.23	2.54
18	2.53	1.87	8.88	N/A	N/A
19	1.89	1.23	6.62	0.27	4.02
20	3.60	1.45	12.66	1.26	9.91

Supplementary Table 1. The MS quantitation consistently gave higher concentrations of ApoA1 in blood compared to Clinical Chemistry data with one exception (subject 14). There is no SD or CV of subject 18 due to issues with the replicate samples. AVG ratio is the ratio of light to heavy peptide. The SD, CV and AVG ratio are from the nLC-MS/MS analysis.

Patient	nLC-MS/MS g/L	Clin Chem g/L	AVG Ratio	SD	CV %
1	0.44	0.87	0.30	0.00	0.33
2	0.46	0.71	0.40	0.01	1.62
3	0.41	0.87	0.20	0.00	0.86
4	0.43	1.14	0.28	0.01	4.38
5	0.45	0.95	0.36	0.01	2.41
6	0.48	1.47	0.49	0.00	0.46
7	0.42	0.57	0.22	0.00	0.36
8	0.45	1.07	0.36	0.00	1.13
9	0.41	0.66	0.21	0.01	5.70
10	0.42	0.71	0.22	0.00	1.64
11	0.41	0.69	0.17	0.01	3.65
12	0.44	0.96	0.31	0.00	0.21
13	0.47	1.34	0.43	0.00	1.25
14	0.41	0.62	0.21	0.00	0.16
15	0.47	1.32	0.44	0.01	3.15
16	0.46	0.96	0.41	0.01	2.02
17	0.46	1.28	0.40	0.01	1.27
18	0.48	1.26	0.49	0.00	0.58
19	0.42	0.81	0.23	0.01	4.58
20	0.46	1.37	0.39	0.00	1.15

Supplementary Table 2. The concentration of apoB measured with the standard clinical chemistry assay was consistently higher than that measured with LC-MS. AVG ratio is the ratio of light to heavy peptide. The SD, CV and AVG ratio are from the nLC-MS/MS analysis.

Patient	nLC-MS/MS g/L	Clin Chem g/mL	AVG Ratio	SD	CV %
1	0.04	0.00063	0.02	0.00	9.81
2	0.05	0.0006	0.06	0.01	17.91
3	0.04	0.00089	0.03	0.01	35.43
4	0.04	0.0026	0.04	0.02	56.52
5	0.05	0.0018	0.06	0.01	13.08
6	0.04	0.0042	0.04	0.01	20.77
7	0.04	0.00094	0.03	0.01	49.01
8	0.04	0.0011	0.03	0.00	3.69
9	0.04	0.0018	0.04	0.02	55.39
10	0.04	0.0012	0.02	0.01	61.35
11	0.06	0.0013	0.10	0.00	4.63
12	0.07	0.0048	0.14	0.14	95.83
13	0.09	0.0017	0.22	0.24	107.73
14	0.05	0.0024	0.08	0.06	74.07
15	0.04	0.0006	0.04	0.03	69.26
16	0.04	0.004	0.02	0.01	30.75
17	0.04	0.0028	0.03	0.00	8.38
18	0.04	0.0045	0.04	0.00	8.77
19	0.04	0.00061	0.02	0.01	27.56
20	0.04	0.0013	0.04	0.03	78.18

Table 3. The concentration of CRP determined with LC-MS/MS was consistently much greater compared to clinical chemistry assay. AVG ratio is the ratio of light to heavy peptide.

Attachment 4.

Supplementary tables from the nLC-MS/MS for fibα and fibβ. Subjects #1-10 are healthy and subjects #11-20 have COPD GOLD stage 1-2. The concentration of fibα was consistently higher than the concentration of fibβ.

Patient	nLC-MS/MS g/L	AVG Ratio	SD	CV %
1	2.71	4.87	0.33	6.80
2	2.08	3.80	0.16	4.25
3	3.99	7.03	0.61	8.62
4	3.41	6.04	0.17	2.77
5	2.51	4.53	0.31	6.80
6	2.21	4.02	0.25	6.29
7	2.74	4.92	0.39	7.92
8	2.37	4.29	0.21	4.79
9	2.46	4.44	0.16	3.71
10	2.60	4.68	0.02	0.39
11	2.03	3.70	0.12	3.17
12	2.05	3.75	0.06	1.54
13	1.86	3.42	0.07	2.10
14	1.49	2.80	0.14	4.96
15	2.97	5.30	0.06	1.06
16	3.12	5.55	0.27	4.77
17	2.26	4.10	0.28	6.90
18	2.76	4.95	0.33	6.63
19	2.20	4.00	0.04	0.98
20	1.76	3.24	0.06	1.95

Table 4. The concentration of fibα has the range of 1.49 g/L – 3.99 g/L across all samples. AVG ratio is the ratio of heavy to light peptide.

Patient	nLC-MS/MS g/L	AVG Ratio	SD	CV %
1	1.63	1.97	0.51	25.65
2	1.77	2.42	0.035	1.44
3	2.27	4.03	1.08	26.83
4	1.72	2.26	0.06	2.88
5	1.58	1.79	0.34	18.86
6	1.74	2.31	0.13	5.53
7	1.64	2.00	0.53	26.52
8	1.72	2.25	0.51	22.71
9	1.52	1.59	0.02	1.16
10	1.56	1.73	0.15	8.60
11	1.63	1.97	0.65	32.90
12	1.64	1.99	0.39	19.52
13	1.47	1.45	0.12	8.45
14	1.43	1.32	0.01	0.58
15	1.65	2.03	0.38	18.56
16	1.74	2.30	0.73	31.49
17	1.67	2.09	0.58	27.64
18	1.56	1.73	0.59	34.43
19	1.56	1.72	0.02	1.33
20	1.68	2.12	0.54	25.59

Table 5. The concentration of fibβ has the range of 1.43 g/L – 2.27 g/L across all samples. AVG ratio is the ratio of heavy to light peptide.