Online Data Supplement

Materials and Methods

The ECLIPSE Cohort

Patients with COPD were recruited according to the inclusion criteria; aged 40-75, current or ex-smokers with >10 pack-year history; a post bronchodilator $FEV_1 < 80\%$ of predicted normal and FEV_1/FVC ratio < 0.7). Smoking (≥ 10 pack-years) and non-smoking (<1 pack-year) control subjects were enrolled if they were aged 40–75 years and had normal lung function (post-bronchodilator $FEV_1 > 85\%$ predicted and $FEV_1/FVC > 0.7$. Study assessments also included pulmonary function measurements, computed chest tomography, biomarkers (in blood, sputum, urine and exhaled breath condensate), health outcome questionnaires and exercise testing (6 minute walk test)[20].

Assessment of disease progression was recorded as changes in spirometry, exacerbation frequency, exercise capacity and assessment of emphysema on CT scanning.

Selection of patients for pancreatic cancer study inclusion included patients with histological diagnosis of adenocarcinoma of the pancreas and locally advanced or metastatic disease, who were either on no chemotherapy or on treatment with gemcitabine or gemcitabine/capecitabine.

Amino Acid Analysis

Chemicals

HPLC grade methanol was purchased from Fisher (Fisher Scientific GmbH, Ulm, Germany) and ammonium formate was purchased from Sigma/Aldrich (MO, USA). The Phenomenex

EZ:Faast LC-MS kit (Phenomenex Inc, Torrence, CA, USA) was used for the preparation of samples for amino acid analysis.

Sample Preparation

The EZ:Faast amino acid analysis procedure consists of a solid phase extraction step followed by derivatisation of the extracted amino acids to form chloroformates. This is followed by a liquid/liquid extraction, with the organic layer removed and taken to dryness under nitrogen. Aliquots of 50μl of serum were prepared in duplicate with the addition of 100μl of the internal standard, which was diluted 1:100 with phosphate buffered solution (PBS). The internal standards solution contained homoarginine, methionine-d3 and homophenylalanine, three components not naturally found in the matrices under investigation. The dried organic extracts were reconstituted in 100μl of a 1:2 (v/v) 10mM ammonium formate in water: 10mM ammonium formate in methanol solution.

Liquid Chromatography

Amino acids were separated using a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters Corporation, Milford, MA, USA). Sample aliquots of 5μl were injected onto a Phenomenex EZ:Faast AAA-MS column (250mm × 2.0mm) held at room temperature. The eluents were of 1mM ammonium formate (Buffer A) and 10mM ammonium formate in methanol (Buffer B). The amino acids were eluted using the following gradient: 0-11 min from 68-83% buffer B, 11-11.01 min 83-68% buffer B and 11.01-13.00 68% buffer B.

A system re-equilibration was performed for 2 minutes prior to each injection. A constant eluent flow of 0.25ml/min was used throughout the analysis. After each injection a strong

needle wash (83:17 methanol:water) and a weak needle wash (10:90 methanol:water) cycle were used to eliminate carry-over.

An amino acid standard mixture was used to create 5-point calibration curves and run at the beginning and end of the serum analysis. A QC sample consisting of the three internal standards, described above was diluted 1:100 and acquired every ten injections to assess the system reproducibility. A pooled sample consisting of 10μl of each sample prepared in the same way as a real sample was injected after every block of ten samples.

Homoarginine was used as the internal standard for the elution of arginine and citrulline. Methionine-d3 was used as the internal standard for the elution of glutamine, citrulline, serine, asparagine, proline-hydroxyproline, 4-hydroxyproline, 3-methylhistidine, 1-methylhistidine, glycine, glycine-proline, threonine, alanine, hydroxylysine, α-aminobutyric acid, sarcosine, aminoisobutyric acid, γ-aminobutyric acid, ornithine, methionine, proline, lysine, aspartic acid, histidine, thiaproline, valine, glutamic acid tryptophan and aminoadipic acid. Homophenylalanine was used as an internal standard for the elution of leucine, phenylalanine, isoleucine, aminopimelic acid, cystathionine, cystine and tyrosine.

Mass Spectrometry

Mass spectrometric data was collected using a Waters Quattro Premier XE Triple Quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) equipped with an electrospray source used in positive ionisation mode. The source temperature was set to 120˚C with a cone gas flow of 50l/hr, a desolvation temperature of 350˚C and a desolvation gas flow of 700l/hr. A capillary voltage of 1000V was applied. Individual cone voltages and collision voltages were optimised for each amino acid and further used to assign the parent and daughter masses to one decimal place. These were used to create individual (multiple reaction monitoring) experiments for each amino acid. A scan time of 0.05 seconds with an inter-scan delay of

0.005 seconds was used for all the analyses. For each amino acid to be considered quantitative at least 15 data points across the peak were required. Analytes detected by MS were compared with authentic standards for confirmation.

LC-MS Data Processing

Raw data were processed and quantified using Waters QuanLynx software version 4.1 (Waters Corporation, Milford, MA, USA) by comparing the peak area with the calibration curves of the internal standards. Responses for each of the amino acids were calculated relative to the internal standards. An integration window extent parameter of 10 was applied.

Statistical Data Analysis

Samples were prepared in duplicate and subjected to statistical analysis. Serum data were normalised to their respective creatinine levels, measured by biochemical assay after first confirming that there were no statistical differences between creatinine concentrations for each of the patient groups and their representative controls (p=0.9, 0.8 and 0.8 for emphysema versus non-emphysema, GOLD IV versus former smoker controls and cachexic versus non-cachexic patients, respectively).

For the ANOVA filtering method as described in Harrington et al., 2005, each of the factor matrices characterised a single variance component, so that the batch effect was described by one matrix and the residual data containing all other variation by another for the same dataset. The duplicate samples were then averaged and the residual factor matrices for each dataset (GOLD IV, cachexia and emphysema) were then subjected to multivariate data analysis.

Supplementary Data Tables

	Smoker Control (n = 30)	GOLD IV (n = 30)	P value
Leucine/glutamine	0.42 ± 0.01	0.37 ± 0.01	0.005
Isoleucine/glutamine	0.22 ± 0.01	0.20 ± 0.01	0.027
Valine/glutamine	0.53 ± 0.02	0.46 ± 0.02	0.018

Supplementary Table 1. Mean (± SEM) ratios of BCAAs to glutamine in smoker control subjects and COPD GOLD IV group.

	P value
1-Methylhisitidne	0.0461*
3-Methylhisitidine	0.0112*
Arginine	0.0190
Aspartic acid	0.0175
β-AIB	0.0065*
GABA	0.0309
Glutamine	7.83 e-7
Lysine	0.0127
Thiaproline	0.0165

Supplementary Table 2. Amino acids significant in the filtered PLS-DA model between smoker controls and the GOLD IV patients. * highlighted amino acids also significant in the unfiltered PLS-DA models.

	EMPH- (n = 21)	EMPH+ (n = 38)	P value
Serine	1.73 ± 0.081	2.10 ± 0.065	0.0009
Glutamine	4.00 ± 0.149	4.95 ± 0.113	<0.0001
Histidine	0.75 ± 0.035	0.93 ± 0.030	0.0004
Arginine	1.71 ± 0.128	2.21 ± 0.086	0.0015
Proline	2.45 ± 0.090	2.80 ± 0.098	0.0230
Asparagine	1.06 ± 0.048	1.19 ± 0.030	0.0212
Aspartic acid	0.25 ± 0.025	0.34 ± 0.024	0.0324
Glycine-proline	2.04 ± 0.316	2.90 ± 0.253	0.0429
Lysine	2.52 ± 0.107	2.85 ± 0.059	0.0044

Supplementary Table 3. Significant mean (± SEM) amino acid concentrations (nmol/mL) in emphysemic and non-emphysemic subjects.

	Cachexic ($n = 30$)	Non-cachexic ($n = 27$)	P value
Serine	2.07 ± 0.087	1.54 ± 0.072	<0.0001
Glutamine	4.03 ± 0.075	3.40 ± 0.073	<0.0001
Phenylalanine	1.00 ± 0.027	0.86 ± 0.023	<0.0001
Histidine	1.06 ± 0.095	0.78 ± 0.026	0.004
Arginine	1.73 ± 0.054	1.46 ± 0.045	0.0002
Proline	2.57 ± 0.071	2.36 ± 0.059	0.0228
Asparagine	1.20 ± 0.034	1.10 ± 0.031	0.0313
Aspartic acid	0.28 ± 0.023	0.20 ± 0.014	0.0064
Citrulline	0.50 ± 0.031	0.40 ± 0.021	0.0067
Hydroxylysine	0.13 ± 0.050	0.54 ± 0.144	0.0134
Aminoadipic acid	0.016 ± 0.004	0.028 ± 0.005	0.0436
Valine	1.96 ± 0.095	1.65 ± 0.066	0.0104

Supplementary Table 4. Significant mean (± SEM) amino acid concentrations (nmol/mL) in cachexic and non-cachexic subjects.

Pancreatic Cancer Cachexia amino acid changes	
DECREASES	INCREASES
β-aminoisobutyric acid	γ-aminobutyric acid**
1-methylhistidine	α-aminobutyric acid
3-methylhistidine	Aspartic acid
Thiaproline	Asparagine
Proline	Phenylalanine
Citrulline	Arginine
Ornithine	Glutamine
Aminoadipic acid	

Supplementary Table 5. Amino acids that significantly distinguish cachexic from non-cachexic patients in the PLS-DA model for pancreatic cancer patients. Highlighted amino acids with corresponding p values, ** $p < 0.01$

	Cachexic ($n = 30$)	Non-cachexic ($n = 27$)	P value
Serine/citrulline	4.01 ± 0.511	2.58 ± 0.039	0.0415
Asparagine/citrulline	0.99 ± 0.103	0.64 ± 0.076	0.0313
Methionine/citrulline	0.11 ± 0.017	0.04 ± 0.008	0.0092
GABA/β-AIB	0.28 ± 0.025	0.14 ± 0.035	0.0055
Methionine/β-AIB	2.83 ± 0.626	0.94 ± 0.191	0.0335
Thiaproline/glutamine	0.02 ± 0.019	0.03 ± 0.022	0.0106
GABA/glutamine (*1000)	10.4 ± 1.085	6.40 ± 0.073	0.0174
GABA/phenylalanine (*1000)	4.76 ± 0.54	2.96 ± 0.318	0.0287

Supplementary Table 6. Significant mean ratios ($\pm$ SEM) of amino acids in cachexic and non-cachexic subjects with pancreatic cancer.

	Ctl/GOLD IV	Emphysema	COPD Cachexia	Cancer cachexia	*Present across studies*
Glutamine	↑ (ns)*	↑ **	↑ **	↑ (ns)*	up
Aspartic Acid	↑ (ns)*	↑ **	↑ **	↑ (ns)*	up
Arginine	↑ (ns)*	↑ **	↑ **	↑ (ns)*	up
Aminoadipic acid	↓ (ns)*	↓ (ns)*	↓ **	↓ (ns)*	down
β-AIB	↑ **	↓ (ns)*	↓ (ns)*	↓ (ns)*	
Proline	↓ (ns)*	↑ **	↑ **	↓ (ns)*	
1-MH	↑ **	↓ (ns)*	↓ (ns)*	↓ (ns)*	
Serine		↑ **	↑ **		
Histidine		↑ **	↑ **	↓ (ns)*	
Asparagine			↑ **	↑ (ns)*	
Sarcosine		↓ (ns)*	↑ (ns)*		
Tryptophan		↓ (ns)*	↑ (ns)*		
3-MH	↑ **				
BCAAs	↓ (ns)*				
GABA				↑ **	

Supplementary Table 7. Amino acids changes across the four studies. (ns) not significant by univariate t-tests, * significant in the multivariate models and ** significant by t-test and in the multivariate models.