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 FEV₁ < 80% of predicted normal and FEV₁/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 FEV₁>85% predicted and FEV₁/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, thiazanine, 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

<table>
<thead>
<tr>
<th></th>
<th>Smoker Control</th>
<th>GOLD IV</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 30)</td>
<td>(n = 30)</td>
<td></td>
</tr>
<tr>
<td>Leucine/glutamine</td>
<td>0.42 ± 0.01</td>
<td>0.37 ± 0.01</td>
<td>0.005</td>
</tr>
<tr>
<td>Isoleucine/glutamine</td>
<td>0.22 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.027</td>
</tr>
<tr>
<td>Valine/glutamine</td>
<td>0.53 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Supplementary Table 1. Mean (± SEM) ratios of BCAAs to glutamine in smoker control subjects and COPD GOLD IV group.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Methylhistidine</td>
<td>0.0461*</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>0.0112*</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.0190</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.0175</td>
</tr>
<tr>
<td>β-AIB</td>
<td>0.0065*</td>
</tr>
<tr>
<td>GABA</td>
<td>0.0309</td>
</tr>
<tr>
<td>Glutamine</td>
<td>7.83 e-7</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.0127</td>
</tr>
<tr>
<td>Thiaproline</td>
<td>0.0165</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>EMPH- (n = 21)</th>
<th>EMPH+ (n = 38)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>1.73 ± 0.081</td>
<td>2.10 ± 0.065</td>
<td>0.0009</td>
</tr>
<tr>
<td>Glutamine</td>
<td>4.00 ± 0.149</td>
<td>4.95 ± 0.113</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.75 ± 0.035</td>
<td>0.93 ± 0.030</td>
<td>0.0004</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.71 ± 0.128</td>
<td>2.21 ± 0.086</td>
<td>0.0015</td>
</tr>
<tr>
<td>Proline</td>
<td>2.45 ± 0.090</td>
<td>2.80 ± 0.098</td>
<td>0.0230</td>
</tr>
<tr>
<td>Asparagine</td>
<td>1.06 ± 0.048</td>
<td>1.19 ± 0.030</td>
<td>0.0212</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.25 ± 0.025</td>
<td>0.34 ± 0.024</td>
<td>0.0324</td>
</tr>
<tr>
<td>Glycine-proline</td>
<td>2.04 ± 0.316</td>
<td>2.90 ± 0.253</td>
<td>0.0429</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.52 ± 0.107</td>
<td>2.85 ± 0.059</td>
<td>0.0044</td>
</tr>
</tbody>
</table>
Supplementary Table 4. Significant mean (± SEM) amino acid concentrations (nmol/mL) in cachexic and non-cachexic subjects.

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

Electronic Supplementary Material (ESI) for Molecular BioSystems
This journal is © The Royal Society of Chemistry 2012
<table>
<thead>
<tr>
<th>Pancreatic Cancer Cachexia amino acid changes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DECREASES</strong></td>
<td><strong>INCREASES</strong></td>
</tr>
<tr>
<td>β-aminoisobutyric acid</td>
<td>γ-aminobutyric acid**</td>
</tr>
<tr>
<td>1-methylhistidine</td>
<td>α-aminobutyric acid</td>
</tr>
<tr>
<td>3-methylhistidine</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Thiaproline</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Proline</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Citrulline</td>
<td>Arginine</td>
</tr>
<tr>
<td>Ornithine</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Aminoadipic acid</td>
<td></td>
</tr>
</tbody>
</table>

**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**
### Supplementary Table 6

Significant mean ratios (± SEM) of amino acids in cachexic and non-cachexic subjects with pancreatic cancer.

<table>
<thead>
<tr>
<th>Amino Acid Ratio</th>
<th>Cachexic ($n = 30$)</th>
<th>Non-cachexic ($n = 27$)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine/citrulline</td>
<td>4.01 ± 0.511</td>
<td>2.58 ± 0.039</td>
<td>0.0415</td>
</tr>
<tr>
<td>Asparagine/citrulline</td>
<td>0.99 ± 0.103</td>
<td>0.64 ± 0.076</td>
<td>0.0313</td>
</tr>
<tr>
<td>Methionine/citrulline</td>
<td>0.11 ± 0.017</td>
<td>0.04 ± 0.008</td>
<td>0.0092</td>
</tr>
<tr>
<td>GABA/β-AIB</td>
<td>0.28 ± 0.025</td>
<td>0.14 ± 0.035</td>
<td>0.0055</td>
</tr>
<tr>
<td>Methionine/β-AIB</td>
<td>2.83 ± 0.626</td>
<td>0.94 ± 0.191</td>
<td>0.0335</td>
</tr>
<tr>
<td>Thiaproline/glutamine</td>
<td>0.02 ± 0.019</td>
<td>0.03 ± 0.022</td>
<td>0.0106</td>
</tr>
<tr>
<td>GABA/glutamine (*1000)</td>
<td>10.4 ± 1.085</td>
<td>6.40 ± 0.073</td>
<td>0.0174</td>
</tr>
<tr>
<td>GABA/phenylalanine (*1000)</td>
<td>4.76 ± 0.54</td>
<td>2.96 ± 0.318</td>
<td>0.0287</td>
</tr>
<tr>
<td></td>
<td>Ctl/GOLD IV</td>
<td>Emphysema</td>
<td>COPD Cachexia</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------</td>
<td>-----------</td>
<td>---------------</td>
</tr>
<tr>
<td>Glutamine</td>
<td>↑ (ns)*</td>
<td>↑ **</td>
<td>↑ **</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>↑ (ns)*</td>
<td>↑ **</td>
<td>↑ **</td>
</tr>
<tr>
<td>Arginine</td>
<td>↑ (ns)*</td>
<td>↑ **</td>
<td>↑ **</td>
</tr>
<tr>
<td>Aminoadipic acid</td>
<td>↓ (ns)*</td>
<td>↓ (ns)*</td>
<td>↓ (ns)*</td>
</tr>
<tr>
<td>β-AIB</td>
<td>↑ **</td>
<td>↓ (ns)*</td>
<td>↓ (ns)*</td>
</tr>
<tr>
<td>Proline</td>
<td>↓ (ns)*</td>
<td>↑ **</td>
<td>↑ **</td>
</tr>
<tr>
<td>1-MH</td>
<td>↑ **</td>
<td>↓ (ns)*</td>
<td>↓ (ns)*</td>
</tr>
<tr>
<td>Serine</td>
<td>↑ **</td>
<td>↑ **</td>
<td>↑ **</td>
</tr>
<tr>
<td>Histidine</td>
<td>↑ **</td>
<td>↑ **</td>
<td>↑ **</td>
</tr>
<tr>
<td>Asparagine</td>
<td>↑ **</td>
<td>↑ **</td>
<td>↑ **</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>↓ (ns)*</td>
<td>↑ (ns)*</td>
<td>↑ (ns)*</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>↓ (ns)*</td>
<td>↑ (ns)*</td>
<td>↑ (ns)*</td>
</tr>
<tr>
<td>3-MH</td>
<td>↑ **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCAAs</td>
<td>↓ (ns)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GABA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.