

# Multiplatform plasma fingerprinting in cancer cachexia: a pilot observational and translational study.

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## Abstract

**Background** Cachexia is a metabolic syndrome that affects up to 50–80% of cancer patients. The pathophysiology is characterized by a variable combination of reduced food intake and abnormal metabolism, including systemic inflammation and negative protein and energy balance. Despite its high clinical significance, defined diagnostic criteria and established therapeutic strategies are lacking. The ‘omics’ technologies provide a global view of biological systems. We hypothesize that blood-based metabolomics might identify findings in cachectic patients that could provide clues to gain knowledge on its pathophysiology, and eventually postulate new therapeutic strategies.

**Methods** This is a cross-sectional observational study in two cohorts of cancer patients, with and without cachexia. Patients were consecutively recruited from routine clinical practice of a General Oncology Department at ‘12 de Octubre’ University Hospital. Selected clinical and biochemical features were collected. Blood metabolite fingerprinting was performed using three analytical platforms, gas chromatography coupled to mass spectrometry (GC–MS), capillary electrophoresis coupled to mass spectrometry (CE–MS), and liquid chromatography coupled to mass spectrometry (LC–MS). Besides, we performed pathway-based metabolite analyses to obtain more information on biological functions.

**Results** A total of 15 subjects were included in this study, 8 cachectic and 7 non-cachectic patients. Metabolomic analyses were able to correctly classify their samples in 80% (GC–MS), 97% (CE–MS), 96% [LC–MS (positive mode)], and 89% [LC–MS (negative mode)] of the cases. The most prominent metabolic alteration in plasma of cachectic patients was the decrease of amino acids and derivatives [especially arginine, tryptophan, indolelactic acid, and threonine, with 0.4-fold change (FC) compared with non-cachectic patients], along with the reduction of glycerophospholipids [mainly lysophosphatidylcholines(O-16:0) and lysophosphatidylcholines(20:3) sn-1, FC = 0.1] and sphingolipids [SM(d30:0), FC = 0.5]. The metabolite with the highest increase was cortisol (FC = 1.6). Such alterations suggest a role of the following metabolic pathways in the pathophysiology of cancer cachexia: arginine and proline metabolism; alanine, aspartate, and glutamate metabolism; phenylalanine metabolism; lysine degradation; aminoacyl-tRNA biosynthesis; fatty acid elongation in mitochondria; tricarboxylic acids cycle; among others.

**Conclusions** These findings suggest that plasma amino acids and lipids profiling has great potential to find the mechanisms involved in the pathogenesis of cachexia. Metabolic profiling of plasma from cancer patients show differences between cachexia and non-cachexia in amino acids and lipids that might be related to mechanisms involved in its pathophysiology. A better understanding of these mechanisms might identify novel therapeutic approaches to palliate this unmet medical condition.

**Keywords** Cancer; Cachexia; Metabolomics; Biomarkers

Received: 7 June 2017; Revised: 4 October 2017; Accepted: 24 October 2017

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## Introduction

Cachexia is a metabolic multifactorial syndrome that affects a large amount of cancer patients, especially in advanced stages of the disease. It is caused by a variable combination of reduced food intake and abnormal metabolism that result in negative balances of energy and protein. By international consensus, cancer cachexia is defined as an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and that leads to progressive functional impairment.<sup>1,2</sup> A variety of tumours (pancreas, stomach, lung, oesophagus, colon, etc.) are associated with a high incidence of cachexia, but not all patients with these tumour types develop cachexia. This disorder may affect to 50–80% of patients with advanced cancer.<sup>3,4</sup> In addition, cachexia might reduce patients' ability to receive, tolerate, and respond to therapy and predict poor outcome independently of other risk factors.<sup>5</sup>

The pathophysiology of cachexia is characterized by a negative energy balance in the body resulting from a combination of inadequate energy intake (anorexia), increased energy expenditure,<sup>6</sup> and systemic inflammation,<sup>7</sup> but abnormal metabolism is the major component.<sup>8–11</sup> All together result in a clinical phenotype characterized by muscle wasting and body weight loss.<sup>12–15</sup> Cancer cells not only produce tumour-specific pro-cachectic factors but also interact with host cells to produce cytokines, which lead to an acute phase response, neuro-endocrine activation, and a chronic state in which catabolism dominates anabolism. Changes in metabolism may be accompanied by anorexia, fatigue, and nausea that, in turn, exacerbate weight loss. This energy imbalance depletes the body energy reserves, especially muscle and adipose tissue.<sup>16,17</sup>

The understanding of the molecular mechanisms of cachexia has increased in the last 10 years.<sup>2,18,19</sup> Conventional management of cachexia is often focused on the use of oral nutritional supplements, and sometimes on the control of a systemic inflammatory response. However, although oral nutritional interventions improve some aspects of quality of life, they do not have an impact mortality.<sup>20</sup> Indeed, cancer cachexia is an unmet medical condition<sup>21,22</sup> and new therapeutic targets or strategies are therefore needed.

High throughput approaches ('-omics') to study diseases are being used to find markers to predict prognosis, diagnosis, and reveal targets for future therapies. Among these, metabolomics has acquired a great interest because the metabolites are the end points of biological processes, and therefore it is assumed that they can reflect all the variables that contribute to the phenotype,<sup>23</sup> thus reflecting upstream biological events such as genetic, transcriptomic, or proteomic alterations, and environmental influences.<sup>24</sup> Metabolomics has quickly risen as a new methodology in the cancer biomarker field.<sup>25</sup>

Despite the powerful possibilities of metabolomics, in a 2012 review about novel findings in cachexia,<sup>26</sup> only one

metabolomics Nuclear Magnetic resonance(NMR)-based study in mice was cited.<sup>27</sup> In another study reported in 2011, 63 metabolites were analysed by <sup>1</sup>H-NMR in random urine samples from cancer patients (different types and stages) to examine sample classification performance according to the cachexia, using eight different standard statistical and machine-learning approaches, differences were shown in the excretion of amino acids and their metabolites.<sup>28</sup> Another study showed the possibilities of metabolomics in humans with pancreatic cancer and cachexia<sup>29</sup> with gas chromatography coupled to mass spectrometry (GC-MS). They found intraday differences in some metabolites such as lactate, different at night but not in the morning. Paraxanthine (caffeine metabolite) appeared associated with cachexia, although the authors recognized that there could be a bias in their results because coffee consumption was not controlled, and some of the patients were strong coffee drinkers.

The hypothesis of the present study is that blood-based metabolomics using a pilot multiplatform approach might identify findings in cachectic patients that could provide clues to gain knowledge on its pathophysiology, and eventually postulate new therapeutic strategies.

## Methods

### *Patient cohorts and samples*

From April 2013 to March 2015, 15 subjects were enrolled in an exploratory, observational, cross-sectional, case-control study, at the Medical Oncology Department of the '12 de Octubre' University Hospital (Madrid, Spain). Eligible subjects were 18 years or older patients with advanced or incurable histologically confirmed malignancy and cachexia (defined as an involuntary weight loss of 5% or more within the previous 6 months).<sup>2</sup> Control subjects were defined as patients with cancer without significant weight loss. Non-eligible patients for this study included those with active infection, uncontrolled diabetes, receiving glucocorticoid therapy, or having esophageal-gastrointestinal obstructions responsible for feeding limitations. Patient characteristics are shown in Table 1.

Blood samples were collected from all the participants, and plasma was separated and immediately stored within 2 h of collection at –80 °C until analysis.

This study was approved by the Ethics Committee of the '12 de Octubre' University Hospital, and it was done in accordance with the Declaration of Helsinki, International Conference on Harmonization, and Good Clinical Practice. Written informed consent was provided by all participating patients.

### *Multiplatform metabolic fingerprinting*

To increase the metabolite coverage, samples were analysed by three platforms commonly employed in metabolomics

**Table 1** Baseline demographic and clinical characteristics

	Cachexia	No-cachexia	P
Overall, N (%)	8 (53.3)	7 (46.7)	
Gender			
Female	1	1	
Male	7	6	0.919
Age, years			
Median (range)	61.5 (36-81)	63.86 (48-80)	0.737
Up to 64	5	5	
65 or higher	2	3	
Relative weight loss in the last 3 months, %	17.59 ± 14.09	1.02 ± 2.7	0.011
BMI, kg/m <sup>2</sup>	20.80 ± 3.37	24.54 ± 2.07	0.025
BSA (DuBois), m <sup>2</sup>	1.67 ± 1.45	1.81 ± 1.21	0.068
CRP, mg/L	5.72 ± 5.63	0.74 ± 0.52	0.041
Serum albumin, g/dL	3.30 ± 0.58	4.07 ± 0.55	0.020
Cortisol, µg/dL	25.34 ± 10.57	13.90 ± 3.14	0.017
Cholesterol (mg/dL)	137.75 ± 42.57	180.29 ± 47.83	0.091
cLDL, mg/dL	77.50 ± 41.41	99.0 ± 43.21	0.343
cHDL, mg/dL	40.77 ± 14.57	58.89 ± 17.75	0.049
Triglycerides, mg/dL	97.13 ± 34.47	111.57 ± 41.79	0.476
Haemoglobin, g/dL	11.50 ± 1.34	11.34 ± 1.67	0.843
Neutrophils	6412.5 ± 3158.9	3685.7 ± 1528.0	0.058
Lymphocytes, No/µL	1262.5 ± 785.5	1400.0 ± 668.3	0.723
NLR	7.45 ± 6.61	3.24 ± 2.09	0.131
Leukocytes, No/ µL	8512.5 ± 3659.6	5428.57 ± 1913.74	0.067
ECOG			
0	0	2(28.6)	
1	6 (75.0)	5 (71.4)	
2	1 (12.5)	0	
3	1 (12.5)	0	0.257
Tumour type, N (%)			
Pancreas	3 (37.5)	1 (14.3)	
Ampulloma	1 (12.5)		
Melanoma	3 (37.5)	1 (14.3)	
Cholangiocarcinoma	1 (12.5)		
Stomach		1 (14.3)	
Oesophageal		2 (28.6)	
Colon		1 (14.3)	
Sarcoma		1 (14.3)	
Tumour spread			
Locoregional	3 (37.5)	2 (28.6)	
Metastatic	5 (62.5)	5 (71.4)	

BMI, Body mass index; BSA, Body surface area; CRP, C-reactive protein; NLR, Neutrophil-to-lymphocyte ratio. Data are mean ± standard deviation.

studies: GC–MS, liquid chromatography–mass spectrometry (LC–MS), and capillary electrophoresis–mass spectrometry (CE–MS), using protocols previously described (Appendix S1). Quality control (QC) samples were prepared by pooling surrogate plasma provided by an external source. QCs were analysed throughout the runs, at the beginning and the end of each analysis and every five sample injections, to check the stability and performance of the system.<sup>30</sup>

### Data treatment

Liquid chromatography–mass spectrometry and CE–MS data processing were performed in MassHunter Profinder software version B.06.00 using Molecular Feature Extraction and Recursive Feature Extraction tools. For GC–MS data treatment, deconvolution and identification were carried out using Agilent MassHunter Unknowns Analysis

version B.07.00. Data obtained in the Unknowns Analysis Tool were aligned in Agilent Mass Profiler Professional version B.12.1 and exported to Agilent MassHunter Quantitative Analysis version B.07.00 to obtain the abundance of the compounds. Data matrix obtained from Profinder (CE–MS and LC–MS data) and Quantitative analysis (GC–MS data) were exported to Excel Software (Microsoft Office 2013) for data filtration. As the QC surrogate was used, features were filtered by selecting the data present in at least 80% of the samples in 1 of the 2 groups: cachexia and controls (Appendix S1).

### Compound identification

Putative identification of features from LC–MS and CE–MS representing significant differences in class separation was performed by searching accurate masses against different

databases available online as METLIN (<http://metlin.scripps.edu>), KEGG (<http://www.genome.jp/kegg/genome.html>), and LIPID MAPS (<http://www.lipidmaps.com>) by the CEU Mass Mediator tool (<http://ceumass.eps.uspceu.es/mediator/>). HMDB (<http://hmdb.ca>) was also considered for additional information. Some compounds putatively identified by LC–MS were confirmed by LC–MS/MS (Appendix S2, Table S1).

### Statistical analysis

Differences between profiles from cachexia and control groups, obtained within each technique, were evaluated with univariate and multivariate (MVDA) data analysis. For univariate data analysis, differences between the two groups were evaluated for each individual metabolite by applying the Mann–Whitney  $U$  test ( $p$ -value  $\leq 0.05$ ), using Matlab (R2015a, MathWorks). For MVDA, principal component analysis and orthogonal partial least squares regression (OPLS-DA) were applied using SIMCA-P+ 12.0 (Umetrics, Umea, Sweden). Significant variables in MVDA were selected according to the Jack-knife confident interval and variance important in projection from OPLS-DA model. Furthermore, in order to avoid the risk of overfitting, the model was validated by cross-validation tool. The percentage of correctly classified samples for each model is described in the Results section.

### Pathway mapping of metabolites

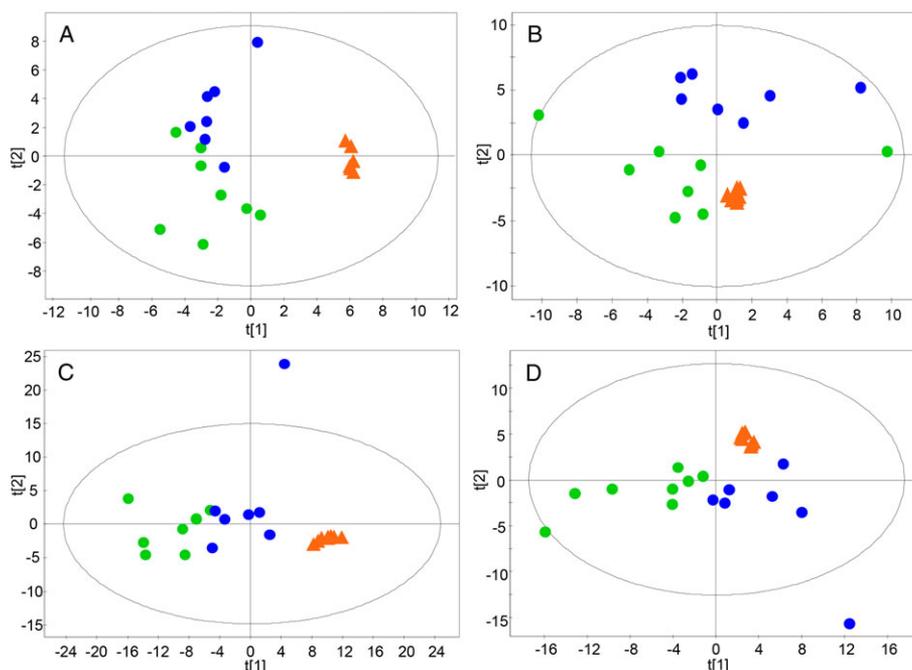
Given that metabolites and enzymes involved in the same biological processes are often dysregulated together in cancer, we believe that pathways-based metabolomics analyses provide more information on biological functions than the metabolites alone. For this purpose, we used MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca/>).<sup>31,32</sup> The Metabolomics Pathway Analysis module combines results from powerful pathway enrichment analysis along with the pathway topology analysis to identify the most relevant pathways involved in our study. Metabolites mapped to pathways are included in Appendix S2, Table S2.

## Results

The diagnosis of cachexia in this study was based on weight loss. Patients with cachexia had significantly lower values in body mass index and body surface area than control patients (Table 1). Clinical biochemical analyses detected higher values of C-reactive protein (CRP) and cortisol in cachectic patients, as well as a significantly lower concentration of plasma albumin and a non-significant decrease in circulating lipids (total cholesterol, triglycerides) (Table 1).

As explained, the metabolite fingerprint was assessed using three analytical platforms, GC–MS, CE–MS, and LC–MS. In GC–MS analysis, data set showed 115 identified

**Figure 1** Principal component analysis-X score plots for all samples in the study. (A) Gas chromatography–mass spectrometry, (B) capillary electrophoresis–mass spectrometry ESI(+), (C) liquid chromatography–mass spectrometry ESI(+), (D) liquid chromatography–mass spectrometry ESI(–). Blue dots, control; green dots, cachexia; orange triangles, quality control.



compounds after alignment and 70 compounds after grouping the different derivatives of the same metabolites. For CE and LC analysis, data set from molecular feature extraction analysis showed 1140 features in positive CE-MS, 1860 and 1062 in positive and negative LC-MS. After recursive feature extraction analysis, feature inspection, and filtering, the data set was reduced to 232, 149, and 95, respectively.

In order to evaluate the performance of analytical platforms reducing data dimensionality, we generated different models by means of principal component analyses (Figure 1). The results show good grouping of QC samples, indicating appropriate performance of the bioanalyses and data quality, and therefore, supporting that the separation between groups are related to biological and not analytical variations. As can be seen, biological samples were slightly separated by the two techniques and one biological outlier was detected in positive and negative LC-MS using Hotelling's  $T^2$  Range. Supervised OPLS-DA were employed for modelling differences between the groups. The applied models showed clear separation of cachexia and control group in all analytical techniques used (Figure 2) with high quality, proven by good values of variance explained ( $R^2$ ) and variance predicted ( $Q^2$ ). To estimate the predictive ability of the OPLS-DA models, cross-validation was performed. Samples were classified correctly in 80% in GC-MS, 97% in CE-MS, 96% in LC-MS (positive mode), and 89% in LC-MS (negative mode). Additionally, the univariate statistical interpretation revealed

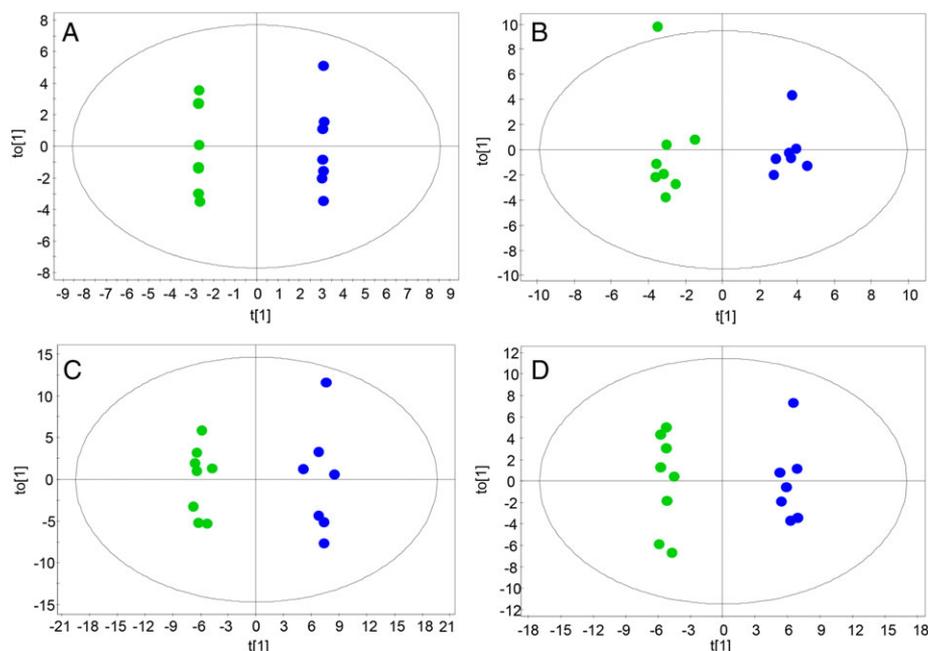
individual significant metabolites, 7 statistically significant features in GC-MS, 24 in LC-MS electrospray ionization (ESI)+, and 58 in LC-MS ESI-.

The results of statistically significant compounds identified and confirmed identification by LC-MS/MS are summarized in Table 2 and Appendix S2. In GC-MS, amino acids were the majority of significant compounds. For CE-MS, the largest group belong to amino acids and amino acid derivatives groups. Finally, in LC-MS, the most populated category corresponded to lysophospholipids, followed by sterol lipids.

The most prominent metabolic alteration in plasma of cachectic patients was the decrease of amino acids and derivatives [especially arginine, tryptophan, indolelactic acid, and threonine, with 0.4-fold change (FC) compared with non-cachectic patients], along with the reduction of glycerophospholipids [mainly lysophosphatidylcholines (LPC(O-16:0) and LPC(20:3) sn-1, FC = 0.1] and sphingolipids [SM(d30:0), FC = 0.5]. The metabolite with the highest increase was cortisol (FC = 1.67).

Additionally, and according to the pathway-based metabolite sets analysis, we identified several pathways with differential features between cachectic and non-cachectic patients in our cohort (Appendix S2, Table S2). Aminoacyl-tRNA biosynthesis is the top-ranked pathway according to enrichment criteria. From a pathway topology analysis perspective, arginine and proline metabolism; alanine, aspartate, and glutamine metabolism; and glycine, serine, and threonine metabolism are the pathways with the highest impact. Amino

**Figure 2** Orthogonal partial least squares regression models. (A) Gas chromatography–mass spectrometry ( $R^2 = 0.998$ ,  $Q^2 = 0.678$ ), (B) capillary electrophoresis–mass spectrometry ESI(+) ( $R^2 = 0.929$ ,  $Q^2 = 0.816$ ), (C) liquid chromatography–mass spectrometry ESI(+) ( $R^2 = 0.827$ ,  $Q^2 = 0.699$ ), (D) liquid chromatography–mass spectrometry ESI(–) ( $R^2 = 0.827$ ,  $Q^2 = 0.703$ ). Blue dots, control; green dots, cachexia.



**Table 2** List of statistically significant compounds identified by a gas chromatography–mass spectrometry (GC–MS), capillary electrophoresis–mass spectrometry (CE–MS), and liquid chromatography–mass spectrometry (LC–MS) with regulation in the cachexia group

Compound name	Molecular formula	Mass (DB)	RT (min)	Mass error (ppm)	p-value	VIP	Jack-knife <sup>b</sup>	FC <sup>c</sup>	Analytical technique	Confirmation	DET
Carboxylic acids and derivatives											
Citric acid	C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>	192.0270	0.81	4	0.014 <sup>a</sup>	1.5	JK	0.52	LC	MS/MS	ESI±
Amino Acids and derivatives											
Glutamine	C <sub>5</sub> H <sub>10</sub> N <sub>2</sub> O <sub>3</sub>	146.0691	14.97	1	0.013	1.1	JK	0.66	CE/GC	Putative	ESI+
Asparagine	C <sub>4</sub> H <sub>8</sub> N <sub>2</sub> O <sub>3</sub>	132.0535	15.86	3	0.0092	1.2	JK	0.68	CE	Putative	ESI+
Arginine	C <sub>6</sub> H <sub>14</sub> N <sub>4</sub> O <sub>2</sub>	174.1117	11.52	1	0.0087 <sup>a</sup>	1.8	JK	0.44	CE	Putative	ESI+
Citrulline	C <sub>6</sub> H <sub>13</sub> N <sub>3</sub> O <sub>3</sub>	175.0957	16.65	1	0.021	1.3	JK	0.54	CE	Putative	ESI+
Ornithine	C <sub>5</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	132.0899	11.09	0	0.0087 <sup>a</sup>	1.7	JK	0.50	CE/GC	Putative	ESI+
Lysine	C <sub>6</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	146.1055	11.17	2	0.0044 <sup>a</sup>	1.9	JK	0.51	CE/GC	Putative	ESI+
Carnitine	C <sub>7</sub> H <sub>15</sub> NO <sub>3</sub>	161.1052	0.75	1	0.041	0.64	—	0.72	LC	MS/MS	ESI+
Methionine	C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub> S	149.0510	16.14	0	0.0044 <sup>a</sup>	1.8	JK	0.52	CE/GC	Putative	ESI+
Phenylalanine	C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>	165.0790	16.61	0	0.028 <sup>a</sup>	1.9	JK	0.56	CE/GC/LC	Putative	ESI+
Tyrosine	C <sub>9</sub> H <sub>11</sub> NO <sub>3</sub>	181.0739	17.02	0	0.028	1.4	JK	0.66	CE	Putative	ESI+
Tryptophan	C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	204.0899	16.54	0	0.0065 <sup>a</sup>	1.8	JK	0.43	CE/GC/LC	Putative	ESI+
Indolelactic acid	C <sub>11</sub> H <sub>11</sub> NO <sub>3</sub>	205.0739	1.01	0	0.024 <sup>a</sup>	1.8	JK	0.41	LC	MS/MS	ESI+
Histidine	C <sub>6</sub> H <sub>9</sub> N <sub>3</sub> O <sub>2</sub>	155.0695	11.75	2	0.013	0.92	JK	0.71	CE	Putative	ESI+
Serine	C <sub>3</sub> H <sub>7</sub> NO <sub>3</sub>	105.0426	15.22	3	0.040	1.0	JK	0.74	CE	Putative	ESI+
Threonine	C <sub>4</sub> H <sub>9</sub> NO <sub>3</sub>	119.0582	15.91	5	0.044 <sup>a</sup>	1.8	JK	0.41	CE/GC	Putative	ESI+
Alanine	C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub>	89.0477	13.95	2	0.013	0.75	JK	0.77	CE	Putative	ESI+
Valine	C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>	117.0790	0.79	0	0.021	1.1	JK	0.55	LC	Putative	ESI+
Glycerophospholipids											
LPC(14:0) sn-2	C <sub>22</sub> H <sub>46</sub> NO <sub>7</sub> P	467.3012	15.14	1	0.040	1.2	JK	0.50	LC	MS/MS	ESI±
LPC(14:0) sn-1	C <sub>22</sub> H <sub>46</sub> NO <sub>7</sub> P	467.3012	15.90	0	0.028	1.2	JK	0.46	LC	MS/MS	ESI±
LPC(16:0) sn-2	C <sub>24</sub> H <sub>50</sub> NO <sub>7</sub> P	495.3325	19.18	2	0.028 <sup>a</sup>	1.8	JK	0.57	LC	MS/MS	ESI±
LPC(16:0) sn-1	C <sub>24</sub> H <sub>50</sub> NO <sub>7</sub> P	495.3325	19.95	2	0.028 <sup>a</sup>	1.8	JK	0.65	LC	MS/MS	ESI±
LPC(O-16:0)	C <sub>24</sub> H <sub>52</sub> NO <sub>6</sub> P	481.3532	21.16	4	0.038 <sup>a</sup>	1.5	JK	0.11	LC	Putative	ESI±
LPC(O-16:1)/LPC(P-16:0)	C <sub>24</sub> H <sub>50</sub> NO <sub>6</sub> P	479.3376	21.17	1	0.045 <sup>a</sup>	1.6	JK	0.70	LC	Putative	ESI±
LPC(18:0) sn-2	C <sub>26</sub> H <sub>54</sub> NO <sub>7</sub> P	523.3638	23.46	0	0.028 <sup>a</sup>	1.8	JK	0.52	LC	MS/MS	ESI+
LPC(18:0) sn-1	C <sub>26</sub> H <sub>54</sub> NO <sub>7</sub> P	523.3638	24.25	2	0.014 <sup>a</sup>	1.6	JK	0.53	LC	MS/MS	ESI+
LPC(O-18:1)/LPC(P-18:0)	C <sub>26</sub> H <sub>54</sub> NO <sub>6</sub> P	507.3689	22.00	0	0.040	1.5	JK	0.69	LC	Putative	ESI±
LPC(18:2) sn-2	C <sub>26</sub> H <sub>50</sub> NO <sub>7</sub> P	519.3325	17.68	0	0.031 <sup>a</sup>	1.5	JK	0.60	LC	MS/MS	ESI±
LPC(18:2) sn-1	C <sub>26</sub> H <sub>50</sub> NO <sub>7</sub> P	519.3325	18.28	1	0.024 <sup>a</sup>	1.4	JK	0.58	LC	MS/MS	ESI–
LPC(20:1) sn-2	C <sub>28</sub> H <sub>56</sub> NO <sub>7</sub> P	549.3794	21.31	4	0.045 <sup>a</sup>	1.5	JK	0.52	LC	Putative	ESI+
LPC(20:1) sn-1	C <sub>28</sub> H <sub>56</sub> NO <sub>7</sub> P	549.3794	24.85	0	0.028 <sup>a</sup>	1.7	JK	0.70	LC	MS/MS	ESI+
LPC(20:2)	C <sub>28</sub> H <sub>54</sub> NO <sub>7</sub> P	547.3638	21.08	0	0.038 <sup>a</sup>	1.5	JK	0.60	LC	Putative	ESI+
LPC(20:3) sn-2	C <sub>28</sub> H <sub>52</sub> NO <sub>7</sub> P	545.3481	19.17	0	0.028 <sup>a</sup>	1.6	JK	0.38	LC	MS/MS	ESI±
LPC(20:3) sn-1	C <sub>28</sub> H <sub>52</sub> NO <sub>7</sub> P	545.3481	20.73	4	0.045 <sup>a</sup>	0.77	JK	0.12	LC	MS/MS	ESI±
LPC(20:4)	C <sub>28</sub> H <sub>50</sub> NO <sub>7</sub> P	543.3325	17.74	4	0.040	1.3	JK	0.63	LC	MS/MS	ESI–
LPC(22:4)	C <sub>30</sub> H <sub>54</sub> NO <sub>7</sub> P	571.3638	21.31	0	0.045 <sup>a</sup>	1.5	JK	0.52	LC	Putative	ESI+
LPC(22:5)	C <sub>30</sub> H <sub>52</sub> NO <sub>7</sub> P	569.3481	20.26	0	0.013	1.2	JK	0.44	LC	MS/MS	ESI+
LPE(16:0) sn-2	C <sub>21</sub> H <sub>44</sub> NO <sub>7</sub> P	453.2855	18.81	0	0.020	1.2	JK	0.67	LC	MS/MS	ESI±
LPE(16:0) sn-1	C <sub>21</sub> H <sub>44</sub> NO <sub>7</sub> P	453.2855	19.59	0	0.028	1.2	JK	0.65	LC	MS/MS	ESI±
LPE(18:0) sn-2	C <sub>23</sub> H <sub>48</sub> NO <sub>7</sub> P	481.3168	19.89	3	0.014 <sup>a</sup>	1.6	JK	0.68	LC	MS/MS	ESI+
LPE(18:0) sn-1	C <sub>23</sub> H <sub>48</sub> NO <sub>7</sub> P	481.3168	22.94	6	0.038 <sup>a</sup>	1.3	JK	0.63	LC	MS/MS	ESI+
LPE(20:0)	C <sub>25</sub> H <sub>52</sub> NO <sub>7</sub> P	509.3481	22.09	1	0.028 <sup>a</sup>	1.8	JK	0.62	LC	Putative	ESI±
LPE(20:3)	C <sub>25</sub> H <sub>46</sub> NO <sub>7</sub> P	503.3012	19.49	7	0.020	1.0	—	0.40	LC	MS/MS	ESI+
PE(24:0)	C <sub>29</sub> H <sub>58</sub> NO <sub>8</sub> P	579.3900	19.89	8	0.014 <sup>a</sup>	1.5	JK	0.68	LC	Putative	ESI–
PE(26:0)	C <sub>31</sub> H <sub>62</sub> NO <sub>8</sub> P	607.4213	24.24	9	0.011 <sup>a</sup>	1.6	JK	0.55	LC	Putative	ESI–
Sphingolipids											
SM(d30:0)	C <sub>35</sub> H <sub>75</sub> N <sub>2</sub> O <sub>6</sub> P	650.5362	31.23	3	0.028	1.3	JK	0.59	LC	Putative	ESI+
SM(d34:1)	C <sub>39</sub> H <sub>79</sub> N <sub>2</sub> O <sub>6</sub> P	702.5676	33.40	0	0.013	1.4	JK	0.78	LC	MS/MS	ESI+
Sterol Lipids											
Cortisol	C <sub>21</sub> H <sub>30</sub> O <sub>5</sub>	362.2093	4.27	1	0.030 <sup>a</sup>	1.3	JK	1.67	LC	Putative	ESI–

VIP, variable importance in the projection; FC, fold change; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamine; PE: phosphatidylethanolamine; LPC: lysophosphatidylcholine; SM: sphingomyelin.

<sup>a</sup>p-value corrected by Benjamini Hochberg (FDR correction).

<sup>b</sup>JK, selected by Jack-Knife confidence intervals estimative, 95 % confidence level.

<sup>c</sup>FC, fold change in the comparison (average in cachexia/average control).

acids implicated in these pathways showed decreased level ranked between 0.41-fold and 0.77-fold in plasma from cachectic patients (Table 2).

Moreover, cortisol is present at higher concentrations in samples from cachectic patients. The increase in cortisol concentrations in samples from cachectic patients was observed

with two independent methodologies: the fold change in cortisol found with our blind approach was 1.67, whereas the respective fold change for cortisol determined with a routine immunoassay method was very similar, 1.82. This adds reliability to the methodology used to find differences in the metabolic profile.

## Discussion

Several studies have tried to correlate different biomarkers with cachexia. Especially oesophageal, pancreatic, and gastrointestinal cancers have shown a positive correlation between high levels of plasma glycerol and free fatty acids, relative weight loss,<sup>33–35</sup> and low serum levels of carnitine.<sup>36</sup> Also, several plasma markers of inflammation have been identified as potential biomarkers of cachexia, including haemoglobin, albumin, CRP, ghrelin, adiponectin, leptin, and IGF-1.<sup>37</sup> Cytokines including IL-1, IL-6, and TNF- $\alpha$  have been associated with cachexia, and clinical trials with antagonists of these molecules are currently under evaluation.<sup>38–40</sup> Most of the evidence about inflammation and cytokines in cachexia has led to the assumption that the major cause for cachexia is a cytokine overproduction.<sup>41</sup>

In our study, we found higher molecular surrogates of inflammation in cachectic patients (CRP, cortisol). Lipid-mobilizing and protein-mobilizing factors produced by the tumour also contribute to activate the inflammatory cascade,<sup>42</sup> which in turn stimulate the release of cortisol and catecholamines from the adrenal glands,<sup>41</sup> subsequently leading to changes in the metabolism of proteins (together with direct cytokine activation of the ubiquitin-mediated proteolytic system), carbohydrates, and lipids.

One of the most relevant results shown in Tables 1 and 2 is the increased concentration of cortisol in cachectic patients, found with two different methodologies. This correlation was already described in 1999, although treatment with an antagonist of glucocorticoid receptors could not abrogate detrimental wasting of muscle.<sup>43</sup> Nevertheless, higher cortisol levels in cachectic patients has not always been detected, because Chauhan *et al.*<sup>44</sup> in a pilot study with nine cachectic patients found only non-significant differences versus nine healthy controls. The type of cancer, the number of patients, the type of treatment, and the sampling hour can be the reasons for this lack of consistency in the elevation of cortisol. This glucocorticoid has a highly relevant role in the complications of age-related degenerative diseases,<sup>45</sup> and the regulation of muscle mass mediated by cortisol.<sup>46</sup> In fact, most of the variations seen in our metabolomics study can be directly related to the increase in cortisol and its relationship with insulin resistance,<sup>47</sup> which is strongly related to cancer cachexia.<sup>48</sup> Noteworthy, given that glucocorticoids might increase appetite,<sup>49</sup> these are frequently offered in routine

clinical practice to cancer patients with cachexia.<sup>50</sup> However, this clinical practice cannot be supported by our findings.

In our study, the most prominent metabolic alteration in plasma of cachectic patients was the decrease of amino acids and derivatives, along with the reduction of glycerolysophospholipids.

Although the studies differ in the change of some amino acids (such as glutamine, histidine, asparagine, arginine, or phenylalanine), our work coincides in the decrease levels of derivatives of histidine, lysine, and tryptophan described by Ubhi *et al.*<sup>51</sup> in patients with cancer related cachexia. Similar to Nezami Ranjbar *et al.*<sup>52</sup> in plasma samples from hepatocellular carcinoma patients vs. patients with liver cirrhosis, we show decreased levels of citric acid ( $p$ -value = 0.014) in plasma from cachectic cancer versus non-cachectic cancer patients.

Protein and amino acid metabolism has been recognized as a key process in cancer cachexia more than 20 years ago, focusing efforts to understand the molecular basis of the problem and identify potential biomarkers.<sup>53,54</sup> The decrease of amino acids showed additional metabolic disorders involving either protein synthesis or amino acid metabolism pathways (Appendix S2, Table S2). Dysregulation of amino acid metabolism and metabolites are early events associated with cancer tumorigenesis. Protein turnover seems to be increased in about 32–35% in cancer patients as a consequence of muscle catabolism; as a result, increased nitrogen excretion is present. In fact, plasma free amino acid profile has been recently proposed as a diagnostic tool for lung cancer.<sup>55</sup> Our multiplatform metabolomics approach has allowed us to see differences (lower signal in cachectic patients) in 13 of the 20 proteinogenic amino acids. Twelve out of the 13 (all but lysine) are glucogenic and could act as substrates for glucose synthesis. Despite the differences in the metabolism for each of them, it is out of discussion that such common trend must be due to alterations in major metabolic pathways that affect simultaneously to all of them, what has been called 'hypermetabolism'. In cachexia, alterations of lactate metabolism (the Cori cycle, i.e. glycolysis in muscle and gluconeogenesis in liver) have been described since the 1970s,<sup>56,57</sup> and increased gluconeogenesis from alanine was found in lung cancer patients with weight-loss vs. weight-stable or healthy controls.<sup>58</sup> In 2006, Bongaerts *et al.*<sup>59</sup> postulated that 'the Cori-cycle, especially the gluconeogenesis, may completely elucidate the cause of cancer cachexia'. Paradoxically, even though there is a dramatic weight loss in these patients, increased proteolysis and increased gluconeogenesis from amino acids are common to diabetes/insulin resistance and metabolic syndrome. This is because insulin resistance and/or metabolic syndrome can be also originated by disturbances in glucocorticoid metabolism, as shown in cases of adrenocortical tumours.<sup>60</sup>

Lower values of triglycerides in plasma of cachectic patients has been shown in other studies,<sup>29,36</sup> but our study

has not been able to detect it. Several studies suggest that an increase in catabolism rather than a defect in anabolism may be more important in triglyceride hydrolysis in these patients.<sup>6</sup> It has been described that cancer patients who are losing weight show an increased turnover of both glycerol and fatty acids compared with normal subjects or cancer patients without weight loss. However, there was no evidence for a decreased level of lipoprotein lipase in the adipose tissue of cancer patients, but there was a two-fold increase in the relative level of mRNA for hormone-sensitive lipase, suggesting an upregulation of triacylglycerol hydrolysis.<sup>6,61</sup> Also, in response to glucose limitation, fatty acid can also be consumed through  $\beta$ -oxidation to provide key substitute energy for cancer cell survival. In our study, there were no big differences in total triglycerides, and the metabolomics approach did not show up differences neither in individual monoglycerides, diglycerides, or triglycerides, nor in free fatty acids. Nevertheless, 19 LPC and 6 lysophosphatidylethanolamines were found lower in cachexia group. To our knowledge, this difference in circulating lysophospholipids has not been previously described associated with cachexia, although Sasagawa *et al.*<sup>62</sup> described changes in the circulating phospholipids associated with the evolution of multiple myeloma, and Muqaku *et al.*<sup>63</sup> showed decreased levels of phosphatidylcholine concentration in blood from metastatic melanoma patients with cachexia.<sup>63</sup> In addition, one of the interesting features of our methodology is that we have been able to find differences in different *sn-1* and *sn-2* lysophospholipids, and therefore, not only phospholipase A2 is involved in these changes. It is noteworthy to mention that among the putatively identified LPC, three have been identified as monoalkylglycerophospholipids [LPC(O-16:0), LPC(O-16:1)/LPC(P-16:0), LPC(O-18:1)/LPC(P-18:0)]. Given that pharmacologically, some monoalkylphospholipids have shown antineoplastic potential,<sup>64</sup> the mechanisms resulting in reduction of such compounds might contribute to cancer progression.

Metabolic regulation is elaborately linked to cancer progression, as proliferating cells demand nutrients for energy production as well as synthesis of genetic materials, proteins, and lipids. Moreover, amino acid, glucose, and phospholipid metabolism can be interconnected through glutaminolysis, a process that supplies carbon and nitrogen resources to the growing and proliferating cancer cells.<sup>65</sup>

In summary, cachexia is multifaceted, and its management will require a multi-modality approach. The complexity of cancer-associated weight loss, coupled with the fact that cancer treatment in general utilizes the paradigm of multi-agent therapy, suggests that cachexia treatment will necessitate more than one concurrent intervention.<sup>66</sup> Also, it is important to recognize the importance of chemotherapy or radiotherapy-induced muscle loss.<sup>67</sup> Intervention is more likely to be effective when given early, before pronounced metabolic abnormalities produce resistance to nutritional intervention.<sup>68</sup> The earlier phase of active anti-cancer therapy,

which frequently achieves good control of tumour, offers a window of opportunity for intervention against malnutrition and, by reducing catabolic drive, against cachexia.

We have described a new model to study metabolomics data for disease identification. Also, pathway-based analysis has significantly enhanced our capacity to explore large-scale omic data, providing an invaluable tool for identifying the modified functionalities involved in this complex disease.

Blood-based metabolomics is a promising method for cachexia research. However, similar to other types of biomarkers, metabolomics biomarker results are difficult to replicate among different studies for a combination of reasons, such as the heterogeneity of the populations and study sizes, variability of the experimental protocols, noise in the metabolomics data, and biological variations in the turnover rates of metabolites.<sup>69</sup> A community effort needs to be made to improve data sharing in order to accumulate statistically well-powered data sets.

A relevant point in cachexia cancer research is its translation to a therapeutic strategy. The lack of complete association between experimental and clinical data likely relates to the fact that not all tumours impinge their effects on the host in the same manner. Finally, patients generally receive systemic antineoplastic therapy, and the interaction of these treatments with the development of cachexia is unknown. Our study was the consequence of the premise that achieving a better understanding of the integrative physiopathology of this complex syndrome may yield yet further novel therapeutic approaches. Development of a successful treatment method will likely require a better understanding of the pathogenesis of cancer cachexia and identification of a dynamic metabolic surrogate marker of pharmacological intervention.

### Limitations of the study

One obvious limitation of this study is the number of samples, but this was an exploratory study in patients in very late stage of the disease. The lack of homogeneity in the types of cancer included has been similarly distributed between both groups. This study design may have resulted in a loss in the identification of tumour type-specific compounds, but this huge data-noise has been able to identify trans-tumoral differences associated with cancer cachexia. Other studies have shown gender differences in metabolic regulation, treatment-related mortality, and progression of cachexia, being the female sex a favourable prognostic factor.<sup>7,70</sup> Our study was not designed to evaluate the effect of gender in the genesis nor the evolution of cancer-related cachexia.

### Ethical standards

The authors certify that they comply with the ethical guidelines for authorship and publishing of the Journal of Cachexia, Sarcopenia, and Muscle.<sup>71</sup> This study was approved by the

Ethics Committee of the '12 de Octubre' University Hospital, and it was done in accordance with the Declaration of Helsinki, International Conference on Harmonization, and Good Clinical Practice. Written informed consent was provided by all participating patients.

## Conflict of interest

None declared.

## Acknowledgements

We would like to thank the patients who participated in this study. This work was supported by Instituto de Salud Carlos III, Fondo de Investigación Sanitaria, and co-financed by the European Regional Development Fund (FEDER-"A way to

make Europe") (grant number PI10/02072); Fundación Médica Mutua Madrileña (grant number MMA10/0018); and the Spanish Ministerio de Economía y Competitividad (grant number CTQ2014-55279-R). The sponsors had no role in study design, data collection, data analysis, data interpretation, writing of the paper, and in the decision to submit the article for publication.

## Online supplementary material

Additional Supporting Information may be found online in the supporting information tab for this article.

**Data S1.** Supporting info item

**Table S1.** Statistically Significant Compounds identified by LC-MS/MS and their characteristic fragments.

**Table S2.** Results from Pathway Analysis.

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