Untargeted metabolomic study of lung cancer patients after surgery with curative intent: possible metabolites of good prognosis, intervention failure and recurrence

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Abstract

Background

Lung cancer (LC) is a leading cause of mortality claiming more than 1.8 million deaths per year worldwide. Surgery is one of the most effective treatments when the disease is in early stages. The study of metabolic alterations after surgical intervention with curative intent could be used to assess the response to treatment or the detection of cancer recurrence.

Methods

In this study, we have evaluated the changes in the metabolomic profile in serum samples (n = 110) from preoperative (PRE) and postoperative (POST) LC patients collected at two different timepoints (1 month - A; 3–6 months - B) with respect to a group of healthy people. An untargeted metabolomic platform based on reversed phase (RP) and hydrophilic interaction chromatography (HILIC), both mounted into an ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS) was applied.

Results

A total of 33 altered metabolites belonging to carboxylic acids, organooxygen compounds, prenol lipids, fatty acyls, glycerolipids and glycerophospholipids classes were annotated comparing all the different studied groups. We found a group of metabolites altered in PRE (gabaculine, DG(14:0/22:1), stearamide, proline and E,e-carotene-3,3'-dione) whose levels returned to those of a baseline control group 3–6 months after surgery. Furthermore, humulinic acid A and 3-galactosyllactose levels may predict an unsuccessful surgery because they remained altered after the intervention in some patients. In addition, tranexamic acid could be studied more exhaustively to be proposed for monitoring the postoperative recurrence of the LC because its levels seem to be close to controls after one month of the intervention, but may differ significantly after 3–6 months.

Conclusions

This study provides unique insights into the metabolic profiles of LC patients after surgery at two different time points with a wide metabolic coverage combining complementary analytical methods.

Background

Lung cancer (LC) is the second most detected cancer in the world representing the first and third leading cause of death in men and women, respectively [1]. Five-year LC survival rates are as low as 4% when patients are diagnosed in advanced stages of the disease, although this rate can increase up to 65% when the disease is detected in early stages and treatments with curative intent are effective [1]. Likewise,
the most appropriate treatment for early stages of non-small cell LC (NSCLC) (stage I and II) is surgery. In spite of surgical intervention, many NSCLC patients need chemotherapy, radiation, target therapy, immunotherapy, or some combination of these treatments to prevent a recurrence [2]. Despite the fact that the survival rate increases with surgical treatment, life expectancy is limited by recurrence, so follow-up after surgery is the norm. In this sense, the identification of biomarkers that provide information on possible metabolic changes before and/or after surgery could improve prevention, early detection, and guide adjuvant or neoadjuvant therapy in order to avoid possible disease recurrence.

Metabolomics is considered a powerful approach investigating the behavior of a wide number of metabolites in a large variety of biological samples including serum. Most metabolomic studies of LC have analyzed biological samples from patients with LC and healthy people to establish metabolic differences between them and to identify potential biomarkers for early diagnosis [3–7]. However, few studies have investigated metabolic alterations in LC patients before and after surgery. Ahmed et al [8] analyzed serum and urine samples from preoperative and postoperative LC patients 4 months after surgery and observed an increase in the levels of lipid and carboxylic acids. Yang et al [9] also described alterations in lipids, fatty acids and amino acids in preoperative and postoperative LC patients 7 days post resection compared to a control group. Similarly, Chen et al [10] reported changes in many metabolites involved in lipid metabolism in LC patients before and after surgical intervention. The metabolomic studies in serum from preoperative and postoperative LC patients have been mainly carried out by using reverse phase (RP) liquid chromatography coupled to a quadrupole time of flight mass spectrometry analyzer (HPLC-QTOF-MS)[8, 10] or gas chromatography mass spectrometry (GC-MS) [10]. However, the use of hydrophilic interaction ultra-high performance liquid chromatography (HILIC) in metabolomics has currently gained growing interest, because it is a complementary technique that allows determining a wide number of polar metabolites. In this sense, only Yang et al [9] have employed this novel analytical technique, to study a limited number of LC patients undergoing surgical resection. Thus, the objective of this work was to determine and identify metabolites that can potentially indicate a good prognosis, failure of the intervention or possible recurrence of LC after surgical intervention with curative intent using metabolomics.

**Methods**

**Study Design**

This study aims to analyze the variations in the global metabolomic profile of patients with NSCLC in early stages that underwent surgery with curative intent. The patients of the study do not have another type of cancer and have not been treated with chemotherapy, radiotherapy or immunotherapy. This is a prospective longitudinal study consisting of two phases: a blood sample collection phase before and after surgery (1 to 6 months after surgery); and a patient follow-up phase in which samples are collected every 3 months for 3 years after surgery, which is still in progress. Control blood samples were collected from healthy volunteers.
Sample Collection

LC and control samples were collected at three different Spanish hospitals. Blood samples were obtained by venipuncture of the antecubital region, after 8 h of fasting, and collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system. The samples were immediately cooled and protected from light for 30 min to allow clot retraction. After centrifugation (2057 g for 10 min) serum samples were aliquoted in Eppendorf® tubes and frozen at −80 °C until analysis.

Samples were divided into 4 groups: a control group of healthy people (CONTROL, 35 samples), a group of preoperative NSCLC patients (PRE, 48 samples), and two groups of postoperative LC patients 1 month (POSTA, 15 samples) and 3–6 months after surgery (POSTB, 17 samples). Clinical data are shown in Table S1 in the Supplementary Material.

Moreover, Table S2 in Supplementary Material describes the number of analyzed samples from patients who underwent surgery and were followed-up after 1 month and 3–6 months of intervention. Some of the patients were not followed-up one month after surgery, and not all patients followed-up one month were followed-up at 3–6 months.

The study was performed in accordance to the principles contained in the Declaration of Helsinki and approved by the Ethical Committee of Andalusian Government (Ethical code num. 1898-N-21). The data of the patients are
anonymized in a database with a hierarchical access control in order to guarantee secure information access.

Reagents

All the solvents used were of HPLC-grade. Methanol, ethanol, acetronitrile, and pyridine were purchased from Aldrich (Steinheim, Germany). Formic acid and ammonium formate were supplied by Merck (Darmstadt, Germany). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK). Palmitic acid-d31 used as internal standard was purchased from Aldrich (Steinheim, Germany).

Sample Treatment

The extraction of metabolites from serum samples was carried out by adding 400 µl of a mixture of methanol/ethanol (1:1 v/v) to 100 µl of serum into Eppendorf® tubes. The samples were vortexed for 5 min at room temperature followed by centrifugation at 2057 g for 10 min at 4°C to eliminate the sediment containing the protein fraction. The supernatant was transferred to another Eppendorf® tube and dried in a fast vacuum system (Thermo Scientific Savant® SPD111 V SpeedVac® Concentrator) at 30°C for 20 min. After that, the remaining pellet was extracted twice with a mixture of acetonitrile/methanol (4:1 v/v) for the extraction of apolar metabolites. For HILIC analysis, samples were extracted by the addition of 400 µl of methanol/water (4:1 v/v). The internal standard palmitic acid-d31 was added to the samples for quality control.

Instrumentation: UHPLC-QTOF-MS

In order to achieve a wide metabolic coverage, a RP and a HILIC coupled to UHPLC were combined. Chromatographic separations were carried out on an Agilent 1290 series coupled to an Agilent 6550 iFunnel Q-TOF-MS equipped with a dual electrospray ionization (ESI) source operated in negative and positive mode (Agilent Technologies, Tokyo, Japan).

For the analysis by RP-UHPLC-QTOF-MS, water (phase A) and acetonitrile (phase B) with 0.1% of formic acid were used as mobile phases following gradient conditions from 5 to 100% of phase B with a total chromatogram time of 30 minutes. The chromatographic separation was carried out in a Zorbax C18, 1.5 µm, 30mmx2.1mm I.D column (Agilent Technologies) in both ionizations, positive and negative modes.

For HILIC-UHPLC-QTOF-MS analysis, mobile phases were composed of 20 mM of ammonium formate and 0.1% of formic acid in water (phase A) and acetonitrile (phase B) with 0.1% of formic acid. The gradient elution was set to 95% down to 45% of B with a total time of 15 minutes. The flow rate was set at 0.4 ml min⁻¹. The chromatographic separation was carried out in Acquity BEH Amide, 1.7 µm, 100 mmx2.1 mm ID column (Waters, Massachusetts, US).
The reference masses used for the mass correction were m/z 121.0509 amu and m/z 922.0098 amu, which were constantly introduced into the system for both ionization modes (positive and negative). The mass range was monitored from m/z 50 to 1100 amu. The QTOF parameters were set to 3 kV for the capillary voltage, 11 L min⁻¹ at 350°C for the drying gas flow rate, and 35 psi for the gas nebulizer. The fragmentor voltage was set to 175 V for both ionization modes. A list containing the most significant features was imported and analyzed with the initial chromatographic conditions using the Agilent MassHunter Data Acquisition software in targeted MS/MS mode with MS/MS scan rate of 1 spectrum s⁻¹. Nitrogen was used as collision gas and several collision voltages were fixed from 10 to 40 V for the fragmentation of compounds. Data were acquired at centroid mode using a scan rate of 1.0 spectrum per second.

Data Processing

For UHPLC-QTOF-MS raw data processing was carried out with Agilent MassHunter Profiler B.10.0 software (Agilent Technologies). To extract the data, Batch Recursive Feature Extraction (RFE) for small molecules wizard from the software was applied. RFE performs two algorithms: First, the Molecular Feature Extraction algorithm (MFE) including extraction, selection of ion species, and charge state was used to find the features in the data set. Second, the initial features were aligned by retention time (RT) and mass, creating a list of unique features through binning. Then, the RT and mass data pairs of the aligned and binning features were used as input criteria to more accurately find the features using the Find by Ion algorithm (FbI). Additional filters such as scoring, integration, and peak filters were also applied to the data set. Table S3 shows the parameters and filters used for positive and negative modes. Moreover, Mass Profiler Professional B.10.0 (Agilent Technologies) was used for the normalization the data set using the internal standard.

Statistical Analysis

For UHPLC-QTOF-MS data processing Mass Profiler Professional B.10.0 (Agilent Technologies) was used for the determination of the most relevant metabolites between groups. For both features determined by RP and HILIC UHPLC-QTOF-MS methodologies, principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were carried out in order to compare the serum metabolomic profiles obtained. PCA plots showed a good clustering of the QCs samples (Figure S1, Supplementary Material) demonstrating the stability and reliability of the metabolomics approach. Table S4 (Supplementary Material) reported the coefficients of variation (CV) of QCs that were used to select those compounds with values lower than 15%. The predictive and class separation parameters R² and Q² of all models built were supplied by the software (Table S5, Supplementary Material). Before performing statistical analysis, the data were submitted to Pareto scaling and logarithmic transformation.

To assess the specificity and sensitivity of the altered metabolites, the values of the areas under the curve (AUC) of the received operator characteristics (ROC) analysis were determined using the Metaboanalyst
AUC values higher than 0.75 were considered of clinical utility in medicine [11].

One-way ANOVA and Tukey test for multiple comparisons were applied using STATISTICA 8.0 from StatSoft. Moreover, a Benjamini–Hochberg FDR correction was also applied to adjust the p-values. The level of statistical significance for all tests was set to \( p < 0.05 \).

Annotation Of Serum Metabolites

The Agilent Qualitative Analysis Workflow MassHunter B.08.00 software was used to annotate the compounds. For this purpose, the workflow “Compound Discovery” and the compound mining “Find by Molecular Features” from the software was applied to the data set. METLIN (http://metlin.scripps.edu) and HMDB (http://hmdb.ca) databases were consulted for the annotation of altered compounds considering a score higher than 97%, which reflects how well the compound matches the mass, isotope pattern, and retention time of the target compound.

Moreover, MS-MS experiments were applied to samples in order to confirm the annotation of some compounds using a QTOF (6550 system, Agilent Technologies) with the same chromatographic conditions as applied for the primary analysis. Ions were targeted by collision-induced dissociation fragmentation on the fly based on the previously determined accurate mass and retention time.

Results

Metabolomic profiles of serum samples from CONTROL, PRE, POSTA and POSTB groups were determined using both methodologies ESI(+/-)-RP-UHPLC-QTOF-MS and ESI(+)-HILIC-UHPLC-QTOF-MS. Figure S2 of the Supplementary Material shows the characteristic metabolomic profiles of the different extract of a human serum sample determined by ESI(+/-)-RP-UHPLC-QTOF-MS and ESI(+)-HILIC-UHPLC-QTOF-MS. Blanks were prepared using the same procedure as samples and analyzed at the beginning and at the end of the batch to ascertain the absence of contamination and artifacts during the UHPLC-QTOF-MS analysis.

PLS-DA showed good classifications between groups in the different organic and aqueous extracts analyzed by ESI (+/-)-RP-UHPLC-QTOF-MS and ESI(+)-HILIC-UHPLC-QTOF-MS (Fig. 1a,b,c and d).

The 2D-PLS-DAs built from any pairwise group comparison (Figure S3, S4, and S5, Supplementary Material) are reported in the Supporting Material showing good discrimination between groups.

A total of 33 altered metabolites (Table S6) were annotated combining HILIC-UHPLC-QTOF-MS (13 metabolites) and RP-UHPLC-ESI(±)-QTOF-MS (20 metabolites) analysis. The levels of 15 metabolites were significantly altered (\( p < 0.05 \)) in LC patients compared to the control group. These metabolites were stearamide (0.38-fold), cerulenin (0.73-fold), DG(14:0/22:1) (2.06-fold), DG(16:0/24:1) (1.68-fold), dihydro-5-(2-octenyl)-2(3H)-furanone (2.23-fold), gabaculine (1.90-fold), 6,10,14-trimethyl-5,9,13-
pentadecatrien-2-one (0.63-fold), E,e-carotene-3,3'-dione (1.79-fold), humulinic acid A (2.05-fold), 3-galactosylactose (1.45-fold), gabapentin (1.71-fold), proline (0.63-fold), glucosylgalactosyl hydroxylysine (1.19-fold), 3-b-galactopyranosyl glucose (1.16-fold) and L-carnitine (1.16-fold).

Moreover, we observed significant postoperative changes in a total of 15 metabolites immediately after surgery in the POSTA group when compared to the control group including 2-ethoxynaphthalene (1.43-fold), 6-(2-carboxyethyl)-7-hydroxy-2,2-dimethyl-4-chromanone glucoside (5.36-fold), butyl ethyl malonate (4.34-fold), cerulenin (0.33-fold), DG(14:0/22:1) (6.13-fold), humulinic acid A (3.9-fold), and talaromycin A (3.62-fold), 1-methylhistidine (0.86-fold), 3-galactosylactose (1.59-fold), argininic acid (1.15-fold), cystine (1.21-fold), glucosylgalactosyl hydroxylysine (1.24-fold), L-carnitine (1.18-fold), N-(1-deoxy-1-fructosyl)leucine (0.91-fold), and proline (0.63-fold).

Long term follow-up (POSTB), revealed 15 metabolites altered when compared to the control group including 2-ethoxynaphthalene (5.28-fold), 4-(3-Pyridyl)-butanoic acid (6.02-fold), 6-(2-carboxyethyl)-7-hydroxy-2,2-dimethyl-4-chromanone glucoside (2.29-fold), butyl ethyl malonate (1.66-fold), cerulenin (0.47-fold), choline (1.97-fold), DG(14:0/22:1) (1.29-fold), humulinic acid A (6.07-fold), talaromycin A (3.60-fold), 1-methylhistidine (0.81-fold), 3-b-galactopyranosyl glucose (1.22-fold), 3-galactosylactose (1.48-fold), argininic acid (1.14-fold), cystine (1.29-fold) and gabapentin (1.63-fold).

Interestingly, glucosylgalactosyl hydroxylysine, L-carnitine and proline were significantly altered before and immediately after surgery, but returned to levels similar to the control group baseline after 6 months of surgery, while dihydro-5-(2-octenyl)-2(3H)-furanone, 3-b-galactopyranosyl glucose and gabapentin were altered before surgery and after 6 months of surgery, but not immediately after surgery. Finally, we found that 6,10,14-trimethyl-5,9,13-pentadecatrien-2-one, DG(16:0/24:1), E,e-carotene-3,3'-dione, gabaculine and stearamide were significantly altered before, but not after surgery. We also found significant differences in the abundance of 2-ethoxynaphthalene, 6-(2-carboxyethyl)-7-hydroxy-2,2-dimethyl-4-chromanone glucoside, butyl ethyl malonate, linalyl propianate, LysoPC(17:0), 3-galactosylactose and 3-b-galactopyranosyl glucose after surgery, but not before the intervention, suggesting metabolic changes related to the intervention itself. Figure 2 represents a Venn diagram showing the number of common and different metabolites in the studied groups. As we can see, four altered metabolites (3-galactosylactose, humulinic acid A, cerulenin, and DG (14:0/22:1) were common in PRE, POSTA and POSTB groups. The results for Venn diagram are shown in Table S7 in Supplementary Material.

Figure 3 shows the abundance of the most significant metabolites in C, PRE, POSTA, and POSTB groups of metabolites determined by RP (Fig. 3a) and HILIC (Fig. 3b) techniques.

The abundance of metabolites LysoPC (17:0), stereamide, 6,10,14-trimethyl-5,9,13-pentadecatrien-2-one (Fig. 3a), proline and tranexamic acid (Fig. 3b) decreased in LC patients while their abundance subsequently increased (POSTB) to control levels. Similarly, the levels of DG(14:0/22:1), SM(d17:1/24:0), gabaculine (Fig. 3a) and L-carnitine (Fig. 3b) increased in LC patients while decreased in POSTB with similar levels to those of the control group. In addition, we observed a gradual recovery in the abundance of SM(d17:1/24:0) and stereamide from POSTA to POSTB groups to control levels. Cerulenin, 6,10,14-
trimethyl-5,9,13-pentadecatrien-2-one, 2-ethoxynaphthalene, 3-b-galactopyranosyl glucose, 3-galactosyllactose, argininic acid, cystine and proline levels were altered before and immediately after surgery. Although the abundance of these metabolites changed during follow up in the POST B group, they were not restored to the control levels. Finally, dihydro-5-(2-octenyl)-2(3H)-furanone, palmitamide, humulinic acid A and 1-methylhistidine were a group of metabolites that continued with the progression of LC after surgery.

The most altered classes of metabolites found in the groups of study were carboxylic acids (18%), organooxygen compounds (13%), prenol lipids (13%), fatty acyls (10%), glycerolipids (5%) and glycerophospholipids (5%) (Figure S7). Concretely, the most altered subclasses of metabolites in PRE, POSTA and POSTB compared to the CONTROL group are shown in Fig. 4. Amino acids, peptides and analogues were the most altered metabolites in the PRE, POSTA and POSTB group (26.7%, 31.3% and 26.3% of the total altered metabolites, respectively).

Alterations in dyglycerides, terpenoids and fatty acyl glycosides pre and post-surgery (Fig. 4), suggested perturbations in lipid metabolism, while alterations in carbohydrates and carbohydrate conjugates suggest post-operative changes in carbohydrate metabolism. Similarly, post-operative alterations in dicarboxylic acids and derivatives also suggest that carbohydrate metabolism was affected by the surgical procedure.

Table S6 includes the AUC values of significant altered metabolites. In this sense, cerulenin (AUC = 0.80), stearamide (AUC = 0.81) and 3-galactosyllactose (AUC = 0.81) showed AUC values higher than 0.75 when comparing preoperative vs postoperative values. Moreover, both cerulenin (AUC = 0.89) and galactosyllactose (AUC = 0.99) showed higher values of AUC when comparing values immediately post-surgery with controls, and at 6 months cerulenin, (AUC = 0.86), tranexamic acid (AUC = 0.81) and galactosyllactose (AUC = 0.93) respectively in POSTB compared to CONTROL metabolites with AUC values higher than 0.75.

Finally, butyl ethyl malonate (AUC = 0.75), galactosyllactose (AUC = 0.83), 3-b-galactopyranosyl glucose (AUC = 0.80), and N-(1-deoxy-1-fructosyl)leucine (AUC = 0.76) presented values higher than 0.75 when compared PRE and POSTA groups, and tranexamic acid (AUC = 0.80) in the comparison between PRE and POSTB. Youden index for each ROC analysis are included in Supplementary Material (Table S8).

We can therefore identify a group of metabolites with potential value in the follow up of LC patients who are surgical candidates. Figure 5 shows the abundance bar graphs of differential metabolites in the study groups. Gabaculine, steramide, DG(16:0/24:1), E,e-carotene-3-3-diona an proline were perturbed in PRE group and non-altered after surgery (Fig. 5a), glucosylgalactosyl hydroxyllysine and L-carnitine (Fig. 5b),
Discussion

In this study, we have evaluated metabolic alterations and annotated metabolites that can have potential value as biomarkers of a good prognosis, failure of the intervention, or LC early recurrence, for patients with LC undergoing surgical resection.

Metabolomic studies of human serum samples from preoperative and postoperative LC patients are limited in the literature \[8–10\]. To our knowledge, this is the first untargeted metabolomic study of serum samples from pre- and postoperative LC patients at two separate time points after surgery (1 month - POSTA and 3–6 months - POSTB). Moreover, the combination of HILIC and RP chromatography provide a new approach since most of the published works reported the use of reversed phase ultrahigh liquid chromatography (RP-UHPLC-MS) or gas chromatography (GC-MS) \[8–10\].

Metabolite Alterations In Pre, Posta, And Postb Compared With The Control Group

We found that the majority of altered metabolites against the control increased in the pre- and postoperative groups at 1 month (POSTA) and 3–6 months after surgery (POSTB) when compared to a control group, including amino acids, fatty acyls, carbohydrates and lipids. Yang et al., reported similar results for amino acids, fatty acids and other specific lipids in preoperative and postoperative LC patient samples \[9\]. In our work, increased levels of gabaculine and gabapentin were found in PRE group. These metabolites are chemical analogues of gamma-aminobutyric acid (GABA), an important inhibitory neurotransmitter in the central nervous system. GABA may affect cancer growth and its receptors may have critical regulatory effects on many kinds of cancers, including LC, modulating cancer cell proliferation \[12\]. It may also exhibit tumor suppression in small airway epithelium and lung adenocarcinoma \[13\]. Several authors have reported significantly elevated expression levels of GABA in non-small cell LC tissues \[12\]. We did not find altered levels of gabaculine in our post-operative samples. However, while gabapentin levels were not significantly altered immediately after surgery in our study, they significantly increased during follow-up. Other amino acid analogues such as N-1-deoxy-1-fructosyl leucine, 1-methylhistidine were not altered preoperatively. Otherwise, they did trend downward after surgery. Proline level decreased before and immediately after surgery, but returned to baseline during the follow-up. Several authors have found diminished levels of amino acids in preoperative LC patient samples \[8\] although other authors have described augmented levels of these metabolites. Alterations in amino acids are related to proliferation and survival of cancer cells under genotoxic, oxidative, and nutritional stress \[14\].

We found increased levels of the carbohydrate 3-galactosyllactose in consonance with data reported by Ahmed et al. They found increased levels of several carbohydrates in serum from preoperative LC
patients [8]. It is well known that tumor cells have an impact on carbohydrate metabolism which in turn is linked to unregulated cellular proliferation, rapid proliferation and metastasis [15]. Alterations in several lipids such as fatty acids, lysophosphatidylcholines, lysophosphatidylethanolamines, phosphatidylcholines, sphingomyelins and glycerides have also been described in preoperative samples of LC patients [8–10]. In our study, diacylglycerides (DGs), fatty acyls, sphingomielyns and prenol lipids were altered in pre-operative samples. Specifically, the abundance of DG(14:0/22:1) and DG(16:0/24:1) was higher in this group. DGs contribute to energy storage, energy metabolism and signal transduction and are components of cellular membranes, which act as building blocks for glycerophospholipids, and as lipid second messengers. Some authors have observed diminished levels of tryacylglycerides (TGs) in preoperative LC patients [9]. Although we did not find altered levels of TGs in our study, the degradation of these metabolites could explain the increase of DGs in preoperative LC serum samples. On the other hand, prenol lipids levels such as linalyl propionate and E,e-carotene-3,3´dione were higher in PRE patients. Prenol lipids are important in health due to their antioxidant effect. In this sense, Yang et al., also found alterations in prenol lipids in preoperative LC patients [9]. Lipids are the main components of biological membranes and signaling molecules needed for proliferation, survival, invasion, metastasis, and interaction with the tumor microenvironment [16]. Concentrations of other lipids such as palmitic amide and glucosylgalactosyl hydroxyllysine also increased preoperatively. We also found increased levels of L-carnitine in the same group. L-carnitine is involved in numerous metabolic pathways including β-oxidation of fatty acids where FAs are broken into acetyl-CoA, which then enters the TCA to aid ATP generation. Accumulated evidence suggests that many cancer cells reprogram FAO and rely on this process for proliferation, survival, drug resistance, or metastasis [17]. In several works, authors have found increased levels of L-carnitine in serum from preoperative LC patients [8, 10].

Altered pre-operative levels of some metabolites when compared with healthy controls could be considered as potential biomarkers. These metabolites could be used as short or long-term controls.

**Metabolites altered in PRE group and non-altered after surgery: possible indication of the good prognosis.**

The metabolites stearamide, DG(16:0/24:1), E,e-carotene-3,3-diona, proline and gabaculine were found altered before surgery in PRE patients, but returned to baseline in both postoperative groups. This finding could indicate that a curative resection could influence the levels of these metabolites in LC patients. Moreover, the analysis of ROC curves showed a good value of AUC for stearamide (AUC = 0.81). Several previous studies considered stearamide as a LC-related metabolite (http://cosbi4.ee.ncku.edu.tw/LCMD/). Moreover, gabaculine, also known as 5-amino-1, 3-hexadienyl-carboxylic acid is considered as an ornithine aminotransferase (OAT) inhibitor that is involved in the suppression of tumor cells proliferation [18]. These metabolites may prove to be biomarkers of a successful resection.

**Metabolites altered in PRE and POSTA groups and non-altered in POSTB: possible indication of the long-term good prognosis**

We found several altered metabolites before and immediately after surgery, but returned to baseline after 3–6 months of surgery. The abundance of glucosylgalactosyl hydroxyllysine, L-carnitine and proline were
significantly different in PRE and POSTA against the control group, but these changes were not significant in the POSTB group. In addition, the metabolites glucosylgalactosyl hydroxyllysine and L-carnitine showed good specificity and sensitivity with AUC values of 0.89 and 0.79 respectively in POSTA groups (Tabla S5). These metabolites may prove to be indications of a good prognosis.

**Metabolites altered in PRE, POSTA and POSTB groups with similar trends: possible indicators of unsuccessful surgery with curative intent.**

DG(14:0/22:1), humulinic acid A and 3-galactosyl lactose were significantly increased in PRE, and remained augmented in POSTA and POSTB groups. On the other hand, cerulenin decreased in the three groups. Alterations in these metabolites persisted after surgery. In addition, cerulenin and 3-galactosyl lactose presented AUC values higher than 0.75 in the PRE, POSTA y POSTB groups (Table S6). Hypothetically, these metabolites could indicate the failure of the surgery. It has been reported that humulones are antioxidant compounds with cancer chemopreventive potential [19]. Regarding 3-galactosyl lactose, the rapid proliferation of cancer cells increases their nutritional requirements, resulting in the high expression of lectin-like receptors on the surface of cancer cells that have strong affinity for mannosyl and galactosyl groups [20]. Finally, cerulenin is a natural inhibitor of fatty acid synthase that induced apoptosis in the human cancer cells [21].

**Metabolites altered in PRE and POSTB groups with similar trend, but comparable with the control group in POST A.**

We found several metabolites altered in the POSTB group when compared with the control group, but they were similar to control in POSTA. This group of metabolites includes tranexamic acid, choline, 4-(3-pyridyl)-butanoic acid and SM(d-17:1/24:0). In this sense, only tranexamic acid (TXA) showed a good value of AUC (0.81) in the ROC analysis. TXA has been reported to prevent blood clot breakdown and thus bleeding since it is an analog of lysine which blocks the interaction with fibrin and the conversion of plasminogen to plasmin [22]. Also, a family of compounds related with 4-(3-pyridyl)-butanoic acid such as 4-hydroxy-4-(3-pyridyl)butanoic acid are considered potent carcinogenic compounds related with tobacco [23]. Moreover, the dysregulation of SM in LC has been extensively reported [24].

However, as these metabolites are not comparable in PRE and POSTB groups, we conclude that they may indicate LC early recurrence.

**Study Limitations**

It is important to consider some limitations of this work. First, the number of samples, especially the number of postoperative patients is relatively small. In addition, samples from the same patients that were collected 3 and 6 months after the intervention were included in order to consider a greater number of samples in the POSTB group. In this sense, to secure the conclusions, a validation study in a larger population would be necessary to ascertain theses new insights. On the other hand, there is a lack of
studies analyzing the impact of surgery and inflammation in the metabolome of healthy people, thus acting as a confounding factor that may be difficult to control in further surgery-based analysis.

Conclusions

In this work, we have applied a combined metabolomic platform based on RP and HILIC chromatography coupled with QTOF to determine altered metabolites in serum samples from preoperative and postoperative LC patients one month and 3–6 months after surgery. Gabaculine, steramide, DG(16:0/24:1), E,e-carotene-3-3-diona, proline glucosylgalactosyl hydroxyllysine and L-carnitine warrant further study as potential biomarkers of a successful resection, while humulinic acid A, 3-galactosyllactose and tranexamic acid need validation as markers of an unsuccessful surgery or recurrence. To the best of our knowledge, this work is the first to address the metabolic changes in serum samples from preoperative and postoperative patients collected at two different time points using the combination of RP and HILIC chromatography in order to provide cast a wide metabolic net including metabolites of different polarities.

Abbreviations

DGs
Diacylglycerides
ESI
Electrospray ionization
GC
Gas chromatography
HPLC
High performance liquid chromatography
LC
Lung cancer
LysoPC
Lysophosphocholine
m/z
mass/charge
MS
Mass spectrometry
NSCLC
Non-small cells lung cancer
PC
Lysophosphocholine
PCA
Principal Component Analysis
PLS-DA
Partial Least Square Discriminant Analysis
Q
Quadrupole
QQQ
Triple Quadrupole
ROC
Receiver Operator Characteristic
SCLC
Small cells lung cancer
SM
Sphingomyelin
TAGs
Triacylglycerides
TOF
Time of flight

Declarations

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Ethics approval and consent to participate

The study was performed in accordance to the principles contained in the Declaration of Helsinki and approved by the Ethical Committee of Andalusian Government (Ethical code num. 1898-N-21). The data of the patients are anonymized in a database with a hierarchical access control in order to guarantee secure information access.

Competing interests

The authors declare that they have no competing interests.
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Additional files

Supplementary Material.

References


**Figures**
Figure 1

3D-PLS-DA scatter plot of a) aqueous extracts determined by ESI(+) -RP-UHPLC-QTOF-MS; b) aqueous extracts determined by ESI(−)-RP-UHPLC-QTOF-MS; c) organic extracts determined by ESI(+) -RP-UHPLC-QTOF-MS; d) polar extracts determined by ESI(+)-HILIC-UHPLC-QTOF-MS. CONTROL: black dots, PRE: green dots, POSTA: blue dots; POSTB: red dots.
Figure 2

Venn diagram showing the common number of metabolites in the different studied groups.
Figure 3

Average abundance heatmap of altered metabolites determined by a) ESI(+/−)-RP-UHPLC-QTOF-MS and b) ESI(+)-HILIC-UHPLC-QTOF-MS.

Figure 4

Most altered subclasses of metabolites when comparing PRE, POSTA and POSTB groups with the control group.
Figure 5

Abundance of altered metabolites after compassion with the control group: a) metabolites altered in PRE group and non-altered after surgery; b) metabolites altered in PRE and POSTA groups and non-altered in POSTB; c) metabolites altered in PRE, POSTA and POSTB groups with similar trend. * AUC values higher than 0.75 compared to CONTROL group.

Supplementary Files

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- SupplementaryMaterial.docx