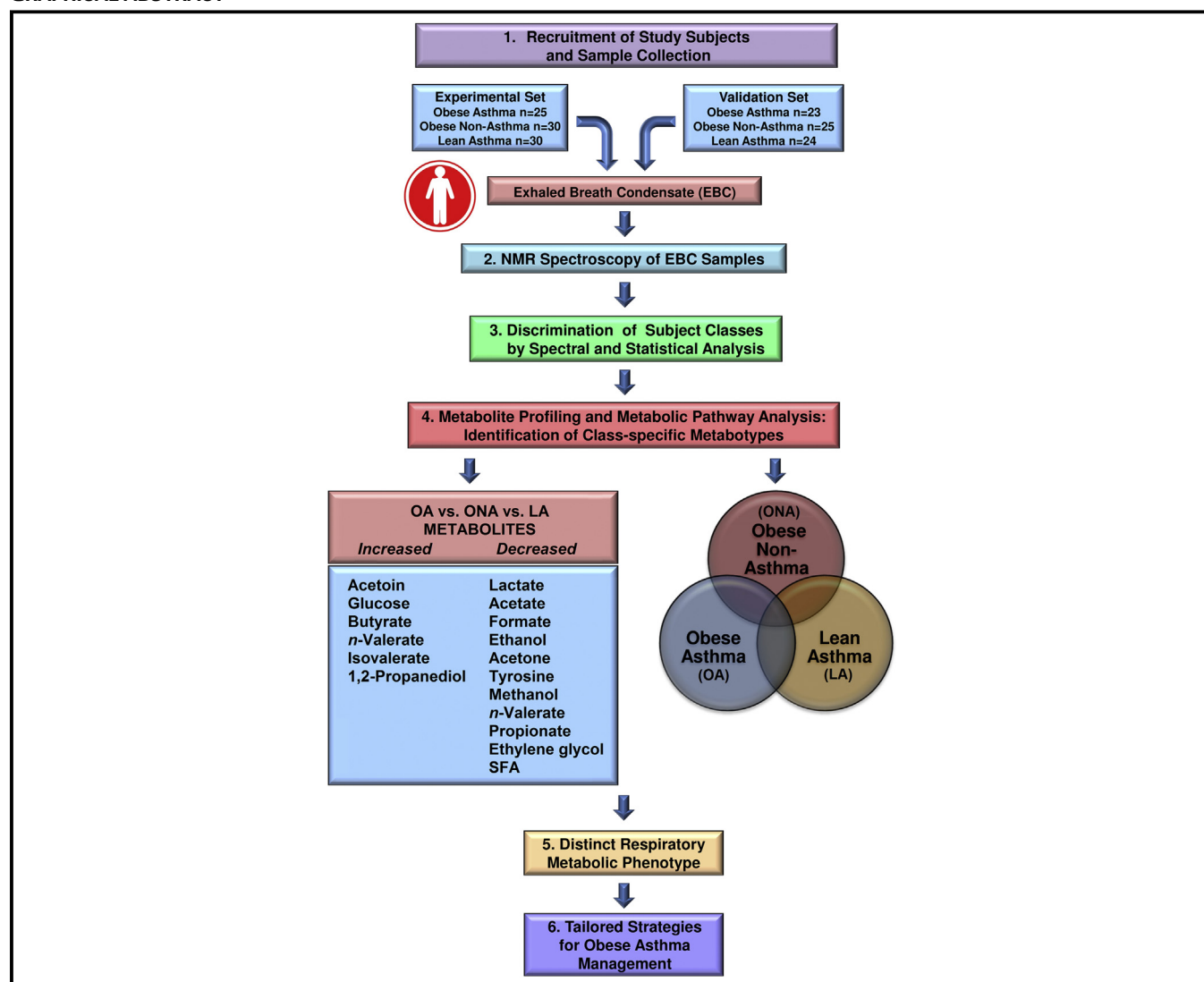


Coexistence of obesity and asthma determines a distinct respiratory metabolic phenotype

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GRAPHICAL ABSTRACT



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Background: Epidemiologic and clinical evidence supports the existence of an obesity-related asthma phenotype. No distinct pathophysiologic elements or specific biomarkers have been identified thus far, but increased oxidative stress has been reported.

Objective: We aimed at verifying whether metabolomics of exhaled breath condensate from obese asthmatic (OA) patients, lean asthmatic (LA) patients, and obese nonasthmatic (ONA) subjects could recognize specific and statistically validated biomarkers for a separate “asthma-obesity” respiratory metabolic phenotype, here defined as “metabotype.”

Methods: Twenty-five OA patients, 30 ONA subjects, and 30 mild-to-moderate LA age-matched patients participated in a cross-sectional study. Nuclear magnetic resonance (NMR) profiles were analyzed by using partial least-squares discriminant analysis, and the results were validated with an independent patient set.

Results: From NMR profiles, we obtained strong regression models that distinguished OA patients from ONA subjects (quality parameters: goodness-of-fit parameter [R^2] = 0.81 and goodness-of-prediction parameter [Q^2] = 0.79), as well as OA patients from LA patients (R^2 = 0.91 and Q^2 = 0.89). The all-classes comparison (R^2 = 0.86 and Q^2 = 0.83) indicated that OA patients possess a respiratory metabolic profile fully divergent from those obtained in the other patient groups. We also identified specific biomarkers for between-class separation, which are independent from clinical bias. They are involved in the methane, pyruvate, and glyoxylate and dicarboxylate metabolic pathways.

Conclusions: NMR-based metabolomics indicates that OA patients are characterized by a respiratory metabolic fingerprint fully different from that of patients independently affected by asthma or obesity. Such a phenotypic difference strongly suggests unique pathophysiologic pathways involved in the pathogenesis of asthma in adult obese subjects.

Furthermore, the OA metabotype could define a strategy for patient stratification based on unbiased biomarkers, with important diagnostic and therapeutic implications. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Obesity, asthma, phenotype, nuclear magnetic resonance, metabolomics, profiling, exhaled breath condensate, biomarkers

Asthma affects more than 350 million subjects worldwide.¹ Both clinical features and underlying pathophysiologic processes indicate that asthma is a heterogeneous disease, phenotypes of which depend on complex genetic traits, inflammatory status, comorbidities, age, time of onset, and demographic characteristics.^{2,3} A phenotype consistently suggested in unbiased cluster analyses is the “asthma-obesity” phenotype,^{4,5} which deserves particular attention given the increasing incidence worldwide of both conditions. Several epidemiologic studies corroborate the association between asthma and obesity,^{6,7} with the latter found to be a risk factor for incident asthma and affecting its severity, control, and medication response.^{8,9}

Several factors (genetic, epigenetic, mechanical, and inflammatory) might contribute to a distinct asthma phenotype occurring in the context of obesity.¹⁰ Obesity is currently viewed as an inflammatory state,^{8,11} and the inflammatory milieu found in

Abbreviations used

BMI:	Body mass index
1D:	One-dimensional
2D:	2-dimensional
EBC:	Exhaled breath condensate
FVC:	Forced vital capacity
GINA:	Global Initiative for Asthma
HSQC:	Heteronuclear single quantum coherence
LA:	Lean asthmatic
NMR:	Nuclear magnetic resonance
OA:	Obese asthmatic
ONA:	Obese nonasthmatic
OPLS:	Orthogonal projections to latent structures
OPLS-DA:	Orthogonal projections to latent structures–discriminant analysis
OSC:	Orthogonal signal correction
PLS-DA:	Partial least-squares discriminant analysis
Q^2 :	Goodness-of-prediction parameter
R^2 :	Goodness-of-fit parameter
ROS:	Reactive oxygen species
SCFA:	Short-chain fatty acid
TOCSY:	Total correlation spectroscopy
TSP:	Sodium 3-trimethylsilyl (2,2,3,3- ² H ₄)propionate

obese asthmatic (OA) patients, both systemically and in the lungs, also supports the existence of a distinct and specific obese asthma phenotype related to the age of onset and the type of underlying inflammation.¹¹ In fact, obesity associated with late-onset asthma displays features that indicate the occurrence of non- T_H2 , oxidative stress–driven inflammation, such as low fraction of exhaled nitric oxide, low eosinophil counts and IgE levels, and poor response to glucocorticoid therapy.^{9,11,12} In this context increased secretion from adipose tissue of the proinflammatory adipokine leptin, as well as reduction in secretion of the anti-inflammatory adipokine adiponectin, might contribute to the development of asthma by skewing airway inflammation toward the mentioned molecular phenotype.^{13,14}

The definition of obesity as a determinant of an independent asthma phenotype will be more conclusive with the identification of reliable biomarkers applicable in diverse clinical settings. Exhaled breath condensate (EBC) is a simple and noninvasive matrix by which several inflammatory biomarkers have been identified within the volatile compartment of the lung epithelial lining fluid,¹⁵ offering potential applications for diagnosis and long-term follow-up. Currently, nuclear magnetic resonance (NMR)–based metabolomics of EBC are widely used for metabolic profiling.^{16,17} It unambiguously recognizes biomarkers that separate healthy subjects from children with asthma,¹⁸ adults with chronic obstructive pulmonary disease,¹⁹ and adults with other pulmonary diseases.^{20–22} We recently reported that obese patients present a specific respiratory metabolic phenotype (“metabotype”) that differentiates them from the corresponding control population of lean asthmatic (LA) patients and nonasthmatic subjects, respectively.^{23,24}

In the present study we investigate whether the additional presence of asthma changes the profile of obese subjects by comparing the metabotype of obese asthmatic (OA) patients with that of LA patients and obese nonasthmatic (ONA) subjects. To further test the hypothesis that asthma in obese subjects holds a specific molecular phenotype, we searched for metabolites that

could characterize the obese asthma metabotype and identify their corresponding metabolic pathways.

Our data indicate that patients with obesity and asthma indeed present a specific respiratory metabotype, which is different from that of patients affected by obesity or asthma alone. This finding strongly indicates the presence of unique pathophysiologic mechanisms, yet to be fully defined, operating in adult asthmatic patients. From the clinical standpoint, our data contribute to the definition of adult asthma in obese subjects as a phenotype, and as such, they might validly assist in developing tailored and effective strategies for asthma managements in these subjects.

METHODS

Study design and study subject enrollment criteria

The study design is presented in Fig 1. All patients, including the external validation set, were prospectively recruited from the Section of Respiratory Medicine, S. Maria della Pietà Hospital. Twenty-five consecutive OA patients and 30 age-matched obese healthy (ONA) subjects were from the weight management program and enrolled for bariatric surgery. Therefore the eligibility criteria followed those for bariatric surgery.²⁵ The enrolled participant age was 30 to 50 years, and the body mass index (BMI) was greater than 30 kg/m² (obesity classes I, II, and III). Excluding criteria included the following comorbidities based on results of laboratory tests and physical examinations and also on self-reported medications: hypertension, diabetes, dyslipidemia, heart disease, sleep apnea syndrome, metabolic disorders, and hypothyroidism/hyperthyroidism. They were diagnosed by using international cutoff values or the use of medication for the specific condition. All patients reported to be nonsmokers with no experience of previous smoking and no occupational or other pronounced exposure to pollutants and did not have a history of chronic respiratory disease. All subjects were free from upper and/or lower airway infection for 4 or more weeks before the EBC collection.

Asthma diagnosis was made based on the results of lung function tests (spirometry or methacholine) performed at the time of evaluation for surgery or was previously physician diagnosed, as for the majority of LA patients. All control (ONA) subjects underwent lung function tests.

No therapy was withdrawn for participation in the study. At enrollment, none of the patients were taking regular corticosteroid therapy, either oral or inhaled, and all were allowed to continue use of salbutamol as needed. EBC collection was performed at least 24 hours after the last use of salbutamol. Finally, patients refrained from food intake for 8 or more hours before the test and from alcoholic drinks for 18 or more hours before EBC collection.

We also enrolled 30 mild-to-moderate, age-matched LA patients (Table I). All subjects provided informed written consent, and the local clinical research ethics committee approved the study.

External validation of the classification models was performed by testing EBC samples obtained from subjects (23 OA patients, 25 ONA subjects, and 24 LA patients) not included in the primary analysis but collected in parallel and according to the above eligibility criteria. Demographic characteristics of all subjects involved in the study are shown in the Results section and Table I.

All OA and LA patients were classified according to Global Initiative for Asthma (GINA) guidelines,¹ considering both the presence of symptoms and either an increase of 12% or greater and 200 mL in FEV₁ after salbutamol or a positive provocation test result (methacholine PC₂₀ <16 mg/mL). Patients without airway reversibility and a negative provocation test result formed the control group. ONA subjects had no history of asthma, atopic disease, respiratory disorders, or other diseases.

Height, weight, and waist and hip circumferences were obtained according to the National Health and Nutrition Examination Survey Web site (<http://www.cdc.gov/nchs/nhanes.htm>). Estimation of body composition was achieved by using the Tanita-BC-418 8-electrode bioelectrical impedance analysis device (Tanita, Arlington Heights, Ill), for which participants were asked to follow all recommended guidelines according to the manufacturer's instructions.

The average age and average BMI of the enrolled and validation subjects are reported in Table I.

Lung function and methacholine challenge tests

Lung function measurements were obtained with a water-sealed spirometer (VMax Encore; CareFusion, San Diego, Calif). The software automatically selects the best of 3 measurements. FEV₁, forced vital capacity (FVC), and FEV₁/FVC ratio were selected as the parameters of interest.

Bronchial hyperresponsiveness was defined as the provocative concentration of methacholine causing a 20% decrease in FEV₁ of 16 mg/mL or less. Test description in full is included in the Methods section in this article's Online Repository at www.jacionline.org.

EBC sampling

EBC samples were collected by using a Jaeger EcoScreen condenser (CareFusion Germany) and randomizing the sequential order of the subject collection, as previously reported.^{19,20} Condensing temperature was measured before and after each sample collection, with $-27.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ as the average value. Samples were stored at -80°C until NMR analysis, which was performed within 1 week from sampling time.

Salivary contamination of the samples was tested by measuring their α -amylase activity. Room temperature remained constant ($24^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$) throughout the sampling period. Possible air contaminants in the collecting room were also monitored. NMR spectra of condensed room air from the collecting device were devoid of signals, confirming the absence of air pollutants (data not shown).

To reduce the risk of contamination by inhaling hospital air, subjects were sampled after 30 minutes of rest in the greenhouse of the Department of Respiratory Medicine, which was shown to be contaminant free, as described above for the collecting room. A full description is included in the Methods section in this article's Online Repository.

NMR sample preparation

EBC samples were rapidly defrosted, and 70 μL of a deuterated water solution (containing a chemical shift reference for ¹H spectra and sodium azide) was added to 630 μL of EBC, reaching 700 μL of total volume. A full description is included in the Methods section in this article's Online Repository.

NMR spectroscopy measurements

All spectra were recorded on a 600-MHz Bruker Avance-III spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a CryoProbe. One-dimensional (1D) proton NMR spectra, 2-dimensional (2D) clean total correlation spectroscopy (TOCSY), and heteronuclear single quantum coherence (HSQC) spectra were recorded by using standard pulse sequences. The spectral positions of the lines ("resonances") in both 1D and 2D TOCSY spectra were referred to the spectral position of the signal originating from 0.10 mmol/L sodium 3-trimethylsilyl [2,2,3,3-²H₄]propionate (TSP), which is assumed to resonate at a δ value of 0.00 ppm.

The spectral positions of the "resonances" in HSQC spectra were referred to the lactate signal (βCH_3), which was assumed to resonate at 1.33 ppm for proton and 20.76 ppm for carbon. Description in full is included in the Methods section in this article's Online Repository.

Spectral and statistical analysis

Before analysis, within-day, between-day, and technical repeatabilities and detection limits were assessed according to published protocols.^{18,20,21}

Discriminating elements in NMR spectra are better appreciated by using multivariate analysis, which carefully identifies hidden phenomena and trends in ensembles of spectra. We carried out a multivariate statistical data analysis using projection methods, as implemented in the SIMCA-P+ 13 package (Soft Independent Modeling of Class Analogy; Umetrics, Umeå, Sweden). To account for the difference in the intensity of NMR signals, we preprocessed the data with Pareto scaling. The model quality was evaluated by using the goodness-of-fit parameter (R^2) and the goodness-of-prediction parameter (Q^2).²⁶

Principal component analysis was first applied to detect EBC metabolite trends and clusterings in an unsupervised (ie, no prior group knowledge is used

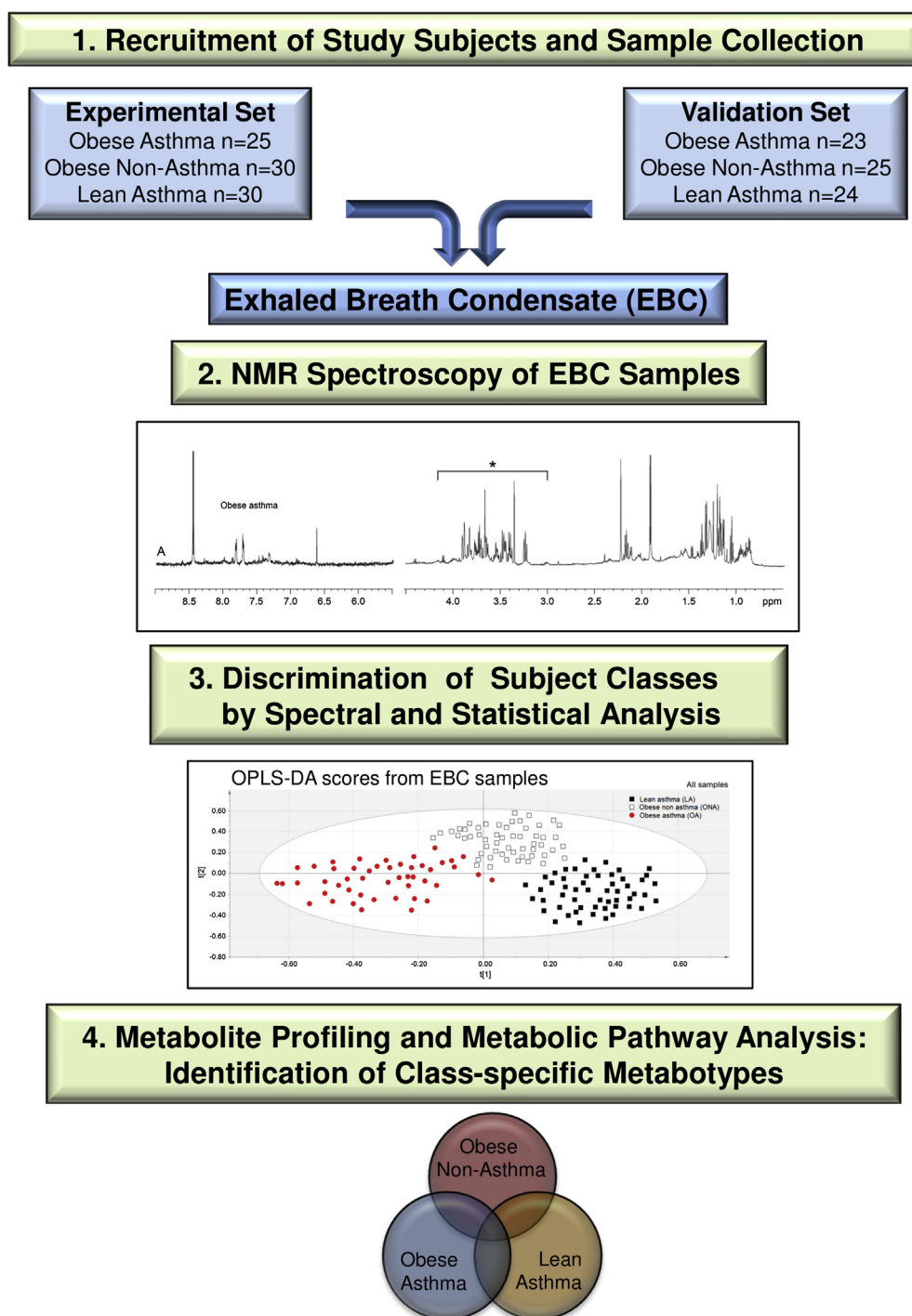


FIG 1. Schematic diagram illustrating the overall study design (see the [Methods](#) section).

in the calculation) manner. However, to better identify clusterings, we tested both orthogonal projections to latent structures (OPLS) and orthogonal signal correction (OSC) routines together with the partial least-squares discriminant analysis (PLS-DA) to verify data fitting and possible data overfitting, which was excluded. The obtained OPLS model turned out to be improved in terms of both predictive and interpretive ability. See the [Methods](#) section in this article's Online Repository for further details and for the equations describing the models.

Demographic data are presented as means $\pm$ SDs after assessing for normality with the D'Agostino-Pearson omnibus normality test. Normally distributed values were compared by using the unpaired Student *t* test. If the

normality test failed, the Wilcoxon-Mann-Whitney test was used. Group differences were explored by means of 1-way ANOVA, followed by *post hoc* multiple comparisons according to the Tukey test. Intraclass correlation analysis was performed for each group to estimate the reliability of single measurements. Significance was defined as a *P* value of less than .05.

Metabolic pathway analysis

Pathway topology and biomarker analysis on selected and more representative metabolites in class separation were applied to the pathway topology search tool in MetaboAnalyst 3.0.²⁷ The centrality was calculated through the

TABLE I. Characteristics of the study population*

	1: ONA subjects	2: LA patients	3: OA patients	P value, 1-2, 1-3, 2-3	4: ONA subjects, validation set	5: LA patients, validation set	6: OA patients, validation set	P value: 4-5, 4-6, 5-6
Anthropometric data								
No.	30	30	25	—	25	24	23	—
Age (y) [†]	38.3 ± 8.9	36.7 ± 7.1	39.0 ± 7.9	.42, .54, .50	36.4 ± 8.9	38.3 ± 9.1	40.4 ± 8.8	.46, .36, .41
Sex (F/M)	18/12	16/14	14/11	—	13/12	11/13	13/10	—
BMI (kg/m ²) [‡]	46.1 ± 10.7	21.6 ± 2.7	45.3 ± 8.9	<.001, .61, <.001	44.7 ± 7.9	20.0 ± 2.6	46.2 ± 9.6	<.001, .59, <.001
Height (m)	1.66 ± 0.07	1.68 ± 0.08	1.62 ± 0.05	.55, .57, .49	1.60 ± 0.06	1.70 ± 0.04	1.68 ± 0.09	.38, .43, .55
Weight (kg)	120.1 ± 14.3	68.6 ± 9.7	126.9 ± 12.1	<.001, .55, <.001	116.4 ± 10.0	67.9 ± 10.0	124.0 ± 18.8	<.001, .53, <.001
Waist circumference (cm)	128.8 ± 15.8	71.9 ± 11.1	121.8 ± 12.1	<.001, .49, <.001	122.9 ± 16.5	70.0 ± 10.9	125.8 ± 17.5	<.001, .74, <.001
Waist-to-hip ratio	0.96 ± 0.09	0.81 ± 0.08	0.98 ± 0.07	.27, .66, .33	0.95 ± 0.09	0.80 ± 0.06	0.97 ± 0.09	.23, .34, .21
Fat-free mass (kg)	57.1 ± 14.5	44.4 ± 8.0	56.1 ± 16.2	<.03, .46, <.04	55.1 ± 13.8	46.1 ± 6.9	56.1 ± 15.2	<.05, .37, <.04
Fat mass (kg)	57.1 ± 13.6	25.2 ± 9.5	58.2 ± 14.9	<.001, .48, <.001	56.9 ± 14.4	24.6 ± 6.3	58.41 ± 16.9	<.001, .58, <.001
Glucose (mg/dL) [§]	94.0 ± 12.7	88.2 ± 7.1	92.1 ± 11.1	.49, .63, .39	93.7 ± 14.3	85.3 ± 6.5	96.1 ± 14.5	.48, .59, .39
Total cholesterol (mg/dL) [¶]	213.1 ± 30.9	196.0 ± 24.4	210.4 ± 38.4	.52, .55, .45	211.1 ± 34.6	186.4 ± 21.0	219.1 ± 39.9	.57, .69, .55
Triglyceride level (mg/dL) [#]	129.7 ± 36.5	118.7 ± 18.4	128.7 ± 39.6	.42, .52, .39	125.1 ± 33.4	111.4 ± 22.3	129.7 ± 44.4	.44, .77, .48
Lung function								
FEV ₁ (% predicted)**	97.8 ± 3.4	79.7 ± 3.2	80.8 ± 4.1	.37, .43, .66	99.1 ± 4.9	77.7 ± 3.7	85.5 ± 4.5	.29, .49, .52
FVC (% predicted)**	94.8 ± 2.9	84.2 ± 2.8	85.2 ± 4.4	.60, .43, .71	97.7 ± 5.1	84.5 ± 2.5	83.2 ± 4.2	.37, .46, .69
PC ₂₀ (mg/mL) ^{††}	—	1.94 ± 1.56 (n = 9)	2.23 ± 1.37 (n = 7)	—, .56, .62	—	1.85 ± 1.32 (n = 7)	2.18 ± 1.43 (n = 6)	—, .58, .66
Asthma classification								
GINA I	—	5	5	—	—	4	3	—
GINA II	—	20	17	—	—	20	19	—
GINA III	—	5	3	—	—	—	1	—

*Data are expressed as numbers or means ± SDs. One-way ANOVA and unpaired *t* tests were used for comparing groups. Significance was defined as a *P* value of less than .05.

[†]Age range: ONA subjects, 30.1-50.6 years; LA patients, 29.3-53.4 years; OA patients, 31.4-48.2 years. *Validation set*: ONA subjects, 29.9-47.3 years; LA patients, 30.3-50.1 years; OA patients, 31.8-49.9 years.

[‡]BMI range: ONA subjects, 33.7-49.4 kg/m²; LA patients, 18.2-22.8 kg/m²; OA patients, 34.2-50.2. *Validation set*: ONA subjects, 34.2-49.7 kg/m²; LA patients, 17.0-24.2 kg/m²; OA patients, 34.2-50.2 kg/m².

[§]Fasting value.

^{||}Multiply milligrams per deciliter by 0.0556 to convert glucose milligrams per deciliter to millimoles per liter.

[¶]Multiply milligrams per deciliter by 0.026 to convert total cholesterol to millimoles per liter.

[#]Multiply milligrams per deciliter by 0.0113 to convert triglyceride milligrams per deciliter to millimoles per liter.

**Prebronchodilator values.

^{††}PC₂₀: provocation concentration of methacholine causing a 20% decrease in FEV₁; *n* is the number of subjects taking the test.

Pathway Impact, a combination of the centrality and pathway enrichment results. Metabolites were selected by evaluating both Variable Importance in Projection values greater than 1 in class discrimination and correlation values $|\rho|$ greater than 0.7. The *Homo sapiens* pathway library was chosen and analyzed by using the Fisher exact test for overrepresentation, whereas the effect of each pathway identified was calculated by using the relative betweenness centrality.

RESULTS

Demographic characteristics of study subjects

Subjects' characteristics and results of dedicated statistical analysis are provided in Table I. FEV₁ and FVC values were reduced in LA and OA patients and were significantly different compared with those of ONA subjects in both the experimental and validation sets. All subjects were normoglycemic, and the total cholesterol and the triglyceride levels were slightly greater than 200 mg/dL and approximately 120 mg/dL, respectively, for obese subjects. The majority of asthmatic patients were classified as GINA II. With the exception of obesity-related data, no

statistical significance was observed between groups. During the study period, there were no changes in medication or occurrences of asthma exacerbations in any of the subjects in the experimental or validation groups.

NMR spectroscopy of EBC discriminates between OA patients and ONA subjects

Fig 2 shows representative NMR profiles (spectra) of EBC samples from an OA patient (spectrum 1A), an ONA subject (spectrum 1B), and an LA patients (spectrum 1C). Metabolites were assigned to lines by resorting to 2D experiments and sample spiking with corresponding standards. No effect of salivary contamination or cleaning solution was observed in EBC profiles. Interestingly, although the absence of saliva in all experimental samples was confirmed by using the α -amylase activity test, we observed strong carbohydrate signals between 3.9 and 3.2 ppm in the OA spectrum (Fig 2, A, region marked with an asterisk).

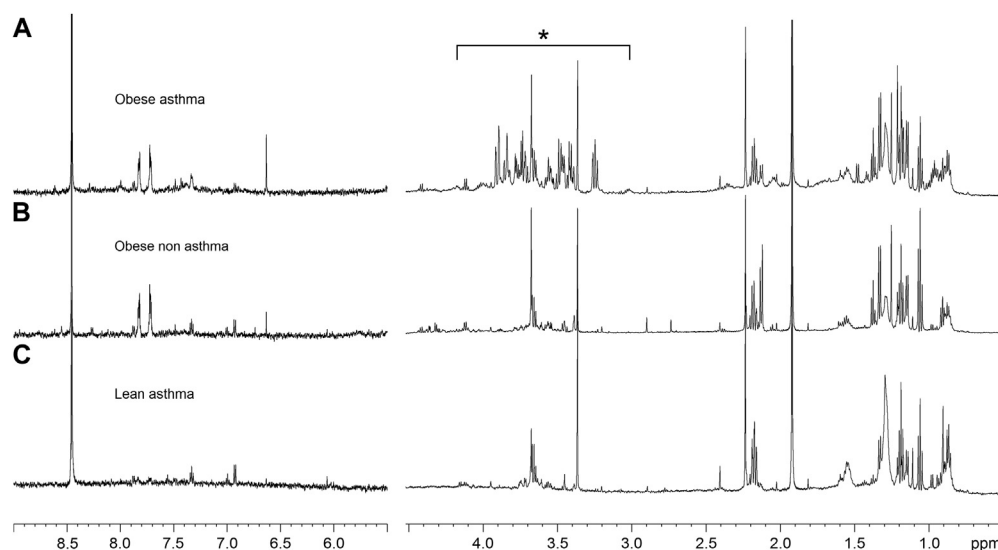


FIG 2. NMR spectra of EBC samples. Representative 1D ^1H spectra of an OA patient (A), ONA subject (B), and LA patient (C). The region between 9.0 and 5.5 ppm has a 32-fold vertical expansion. The region marked with an asterisk in Fig 2, A, indicates strong carbohydrate signals between 3.9 and 3.2 ppm in the OA spectrum (see the Results section). All signals were assigned to single metabolites by resorting to 2D NMR experiments and referring to published data on metabolite chemical shifts. Absorption (related to the intensity) is plotted on the y-axis, and magnetic field strength is plotted on the x-axis, which usually ranges from 0 to 12 ppm.

Statistical analysis of EBC profiles from OA patients and ONA subjects (Fig 3) differentiated the 2 groups, with strong regression (95%, $P < .002$; Fig 3, A). The ONA scores are clearly separated from those of OA samples and are described by high-quality parameters ($R^2 = 0.81$ and $Q^2 = 0.79$). In Fig 3, A, the labels $t[1]$ and $t[2]$ along the axes represent the scores (representing the first 2 partial least-squares components) of the model, which are sufficient to build a satisfactory classification model. For OA patients, a dispersion is observed along the $t[1]$ dimension. EBC samples of OA patients present, with respect to ONA subjects, an increase in glucose, butyrate, and acetoin levels and a decrease in formate, tyrosine, ethanol, ethylene glycol, methanol, *n*-valerate, acetate, saturated fatty acids, and propionate levels. The different levels are shown by the S-line plot of Fig 3, B, which reports the loadings values (ie, the weighting coefficients for each metabolite) plotted against the spectral position (ppm) as a variable. Because each bucket (ie, one of the sequentially integrated spectral regions) is normalized to the total spectrum area (see the Methods section), peaks in opposite directions suggest that variations do not originate from dilutional effects.

The OA-ONA model was validated by using an EBC sample set obtained from subjects not included in the primary analysis (validation set of 23 OA patients and 25 ONA subjects, Table I) by using the procedure described for the experimental set. The model correctly identified 21 of 23 OA patients (91% accuracy) and 24 of 25 ONA subjects (96% accuracy, 4% false-positive results) with a sensitivity of 91% and a specificity of 96%. Fig E1, A, in this article's Online Repository at www.jacionline.org reports the scores plot of the ONA and OA validation samples. Both scores are clearly separated, presenting high-quality parameters ($R^2 = 0.83$ and $Q^2 = 0.87$). The same behavior was observed by combining the 2 sets to increase the number of patients. Fig E2, A, in this article's Online Repository at www.jacionline.org shows that ONA subjects and OA patients

are well separated and that the model obtained for the experimental set is actually valid ($R^2 = 0.89$ and $Q^2 = 0.91$). The metabolites that bring about the class separation are identical to those observed in the experimental set, as reported in Fig 3, B, and in the summary (experimental and validation groups) comparison shown in Fig E3 in this article's Online Repository at www.jacionline.org.

NMR spectroscopy of EBC further discriminates OA patients from LA patients

Notably, NMR profiling of OA patients is also different from that obtained from LA patients' samples, with the model showing a strong regression (95%, $P < .0004$; Fig 4, A). The scores of the 2 classes are well separated, with high-quality parameters ($R^2 = 0.91$ and $Q^2 = 0.89$) and no interference from other variables. The tight clustering observed in this comparison, in which both patient groups are affected by asthma, suggests that the metabolic heterogeneity noted in the OA-ONA model (Fig 3, A) might reflect the heterogeneity of asthma in comparison with a control population. Between-class differences were obtained from the related S-line plot (Fig 4, B), which showed that, when compared with LA patients, OA patients present an increase in glucose, *n*-valerate, acetoin, isovalerate, and 1,2-propanediol levels and a decrease in formate, ethanol, methanol, acetone, propionate, acetate, lactate, and saturated fatty acid levels (also listed in Fig E3).

As for the previous OA-ONA comparison, results obtained in the experimental group for the OA-LA comparison were tested in the validation set. The model correctly identified 21 of 23 OA patients (90% accuracy) and 22 of 24 LA patients (94% accuracy), with a sensitivity of 90% and a specificity of 94% (score plots shown in Fig E1, B). The OA scores are clearly separated from LA scores, with the model presenting high-quality parameters ($R^2 = 0.85$ and $Q^2 = 0.81$). Fig E2, B,

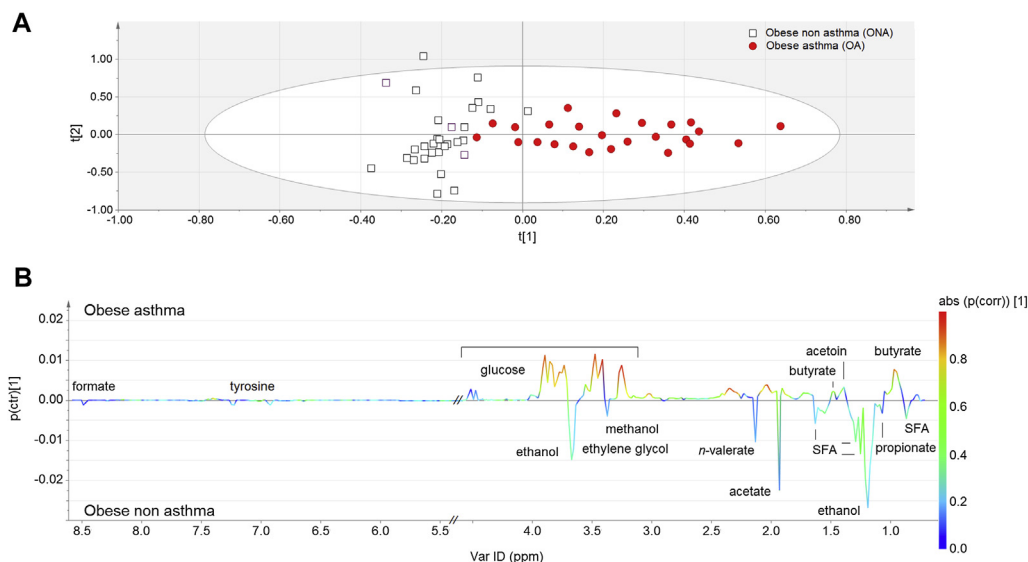


FIG 3. OPLS-DA (see the [Methods](#) section) of EBC samples: OA patients versus ONA subjects. **A**, Score plot showing the degree of separation of the model between OA patients (*red circles*) and ONA subjects (*white squares*). The labels *t[1]* and *t[2]* along the axes represent the scores (the first 2 partial least-squares components) of the model, which are sufficient to build a satisfactory classification model. **B**, S-line plot between 8.5 and 0.5 ppm corresponding to the Fig 3, A, model between OA patients and ONA subjects. Positive signals correspond to metabolites that present an increased concentration in OA patients, whereas negative signals correspond to those that show a decreased concentration in OA patients with respect to ONA subjects. The buckets (ie, the sequentially integrated spectral regions; see the [Methods](#) section) are labeled on the *x-axis* according to metabolite assignment with variable identity. The *y-axis* *p[ctr][1]* indicates the loading value for each variable according to the centering, whereas *abs(p[corr])[1]* refers to the absolute correlation value.

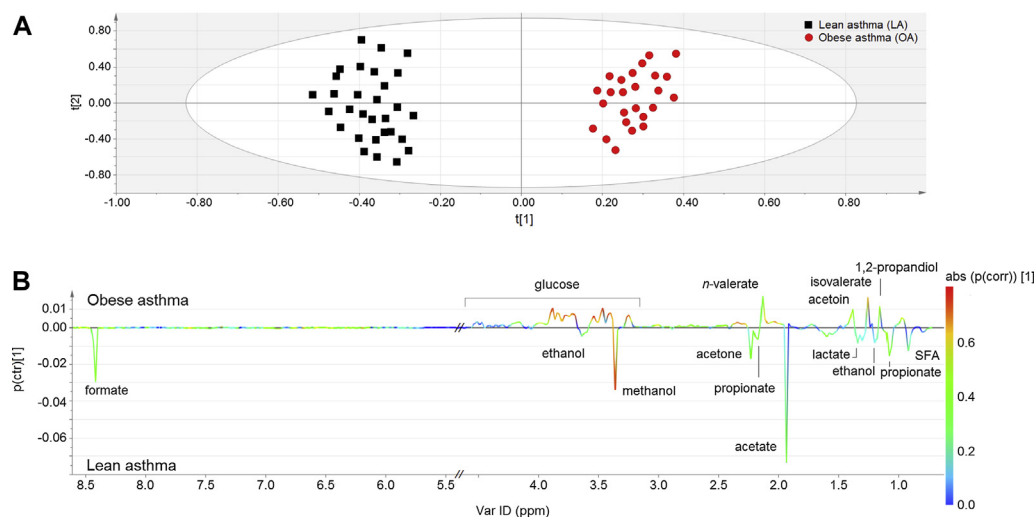


FIG 4. OPLS-DA of EBC samples: OA patients vs LA patients. **A**, Score plots showing the degree of separation of the model between OA patients (*red circles*) and LA patients (*black squares*). **B**, S-line plots between 8.5 and 0.5 ppm, corresponding to the model shown in Fig 4, A. Positive signals correspond to metabolites that present an increased concentration in OA patients, whereas negative signals correspond to those that present a decreased concentration in OA patients with respect to LA patients. Labels on axes are as described for Fig 3, A and B.

shows that OA and LA scores are clearly separated also in the combined sets of patients ($R^2 = 0.91$ and $Q^2 = 0.90$), and the metabolites responsible for the class separation are, as in the OA/ONA comparison, identical to those reported for the experimental set (Fig 4, B, and see Fig E3).

EBC profiling of all classes indicates the OA group as fully separated from the ONA and LA groups

Comparative analysis among all 3 experimental groups yielded a strong regression model (95%, $P < .0004$; Fig 5, A), with high-quality parameters ($R^2 = 0.86$ and $Q^2 = 0.83$). All classes are well

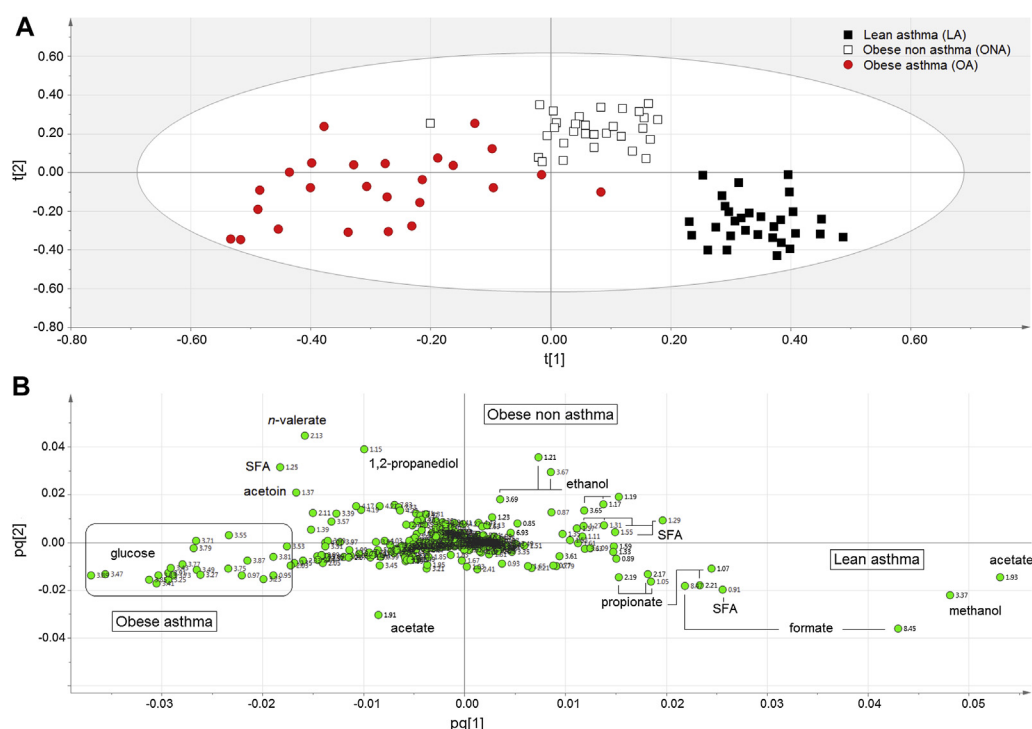


FIG 5. OPLS-DA of EBC samples: OA patients versus ONA subjects versus LA patients. **A**, Score plot showing the degree of separation of the model between OA patients (red circles), ONA subjects (white squares), and LA patients (black squares). **B**, Loading plot associated with the OPLS-DA analysis reported in Fig 5, A, showing the metabolites responsible for between-class differences. Numbers refer to buckets' chemical shifts (spectral positions), and the discriminating metabolites are explicitly labeled. The $pq[1]$ and $pq[2]$ values refer to the weight that combines the X and Y loadings (p and q).

separated, and score clusterings of LA patients and ONA subjects indicate that patients characterized by a single condition (asthma or obesity) are homogeneously distributed. Despite the scattering of OA patients, no subgroups caused by demographic and/or technical parameters were observed in this set.

Metabolites responsible for between-class differences, as identified with chemical shift values, are shown in the loading plot of Fig 5, B; are listed in Fig E3; and correspond to those recognized in the above 2-class comparisons.

Results were replicated by using the validation set. The scores of OA patients, ONA subjects, and LA patients were all well separated (see Fig E1, C), with high-quality parameters ($R^2 = 0.85$ and $Q^2 = 0.85$). Identical results were obtained on combining all sample sets (see Fig E2, C; $R^2 = 0.91$ and $Q^2 = 0.91$). Metabolites responsible for between-class differences are indicated in Fig E3 and correspond to those found for the experimental set of samples.

Pathway topology analysis

To better understand the biological relevance of the data, we investigated the metabolic pathways in which the differently regulated molecular species are involved using MetaboAnalyst 3.0 software. The found pathways are depicted in Fig 6, which reports the impact of each pathway versus P values.

Using the discriminating metabolites, we unveiled 23 and 17 potentially involved metabolic pathways in OA and LA patients, respectively, which appear to be significantly dysregulated.

Accordingly, comparing the OA patients versus ONA subjects, we extrapolated methane ($P = 3.98 \times 10^{-5}$; impact, 0.56), glyoxylate and dicarboxylate ($P = 2.24 \times 10^{-4}$; impact, 0.54), and pyruvate ($P = 3.16 \times 10^{-5}$; impact, 0.50) metabolisms as the most probable. For the OA patient versus LA patient comparison, we obtained the same pathways but with different order and parameters, with pyruvate ($P = 2.00 \times 10^{-7}$; impact, 0.64), methane ($P = 6.31 \times 10^{-4}$; impact, 0.56), and glyoxylate and dicarboxylate ($P = 1.58 \times 10^{-6}$; impact, 0.54) as the most likely divergent metabolisms. This is due to different concentration variations of the involved metabolites in the 2 comparisons. Therefore, although the metabolites and, consequently, the pathways are the same, their different regulation makes the OA patient versus ONA subject and LA patient versus OA patient comparisons theoretically different. The found pathways are classified as energy metabolism (methane) and carbohydrate metabolism (glyoxylate and dicarboxylate, pyruvate) and might contain molecular species potentially relevant as biomarkers for specific pathophysiological processes within the respiratory metabolome.

Correlations

We found no correlation between metabolomic data and spirometric results in any study group. In the ONA and OA groups, there was no correlation between EBC metabolites and the obesity-related parameters. In asthmatic patients (LA and OA patients) there was no correlation between metabolomic profiles and the occasional use of salbutamol (see the Methods section).

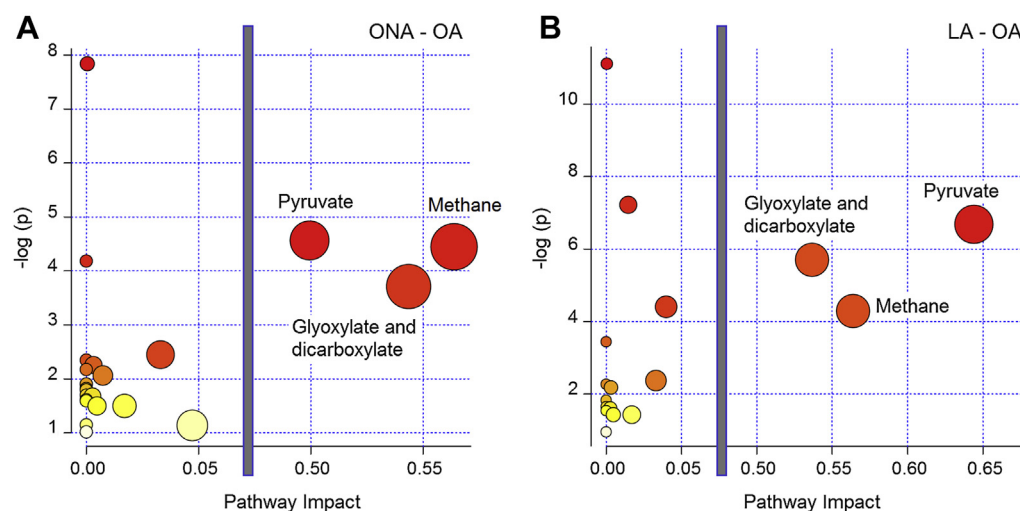


FIG 6. MetaboAnalyst pathway impact based on selected and more representative metabolites responsible for the class separation (see the [Methods](#) section). Circles represent all 23 (**A**; ONA subjects vs OA patients) and 17 (**B**; LA patients vs OA patients) metabolic pathways potentially involved in class separation. The vertical bar in each pathway highlights the 3 metabolisms with higher impact located on the right side of the bar. Methane, glyoxylate/dicarboxylate, and pyruvate were identified in both comparisons but with significantly different *P* values and impact parameters (see the [Results](#) section).

No correlations were found between metabolites and any of the anthropometric parameters (listed in [Table I](#)). Adding the experimental and validation sets of subjects did not reveal any correlation between discriminating metabolites and clinical parameters.

DISCUSSION

We have shown that patients affected by asthma associated with obesity express a respiratory metabolic phenotype (“metabotype”) that is fully different, not summatory, with respect to those separately characterizing patients with either asthma or obesity alone. This novel finding builds on our previous studies on EBC, which identified obese metabolic profiles that were fully separated from those of healthy control subjects.²³ In the present study application of NMR-based metabolomics to EBC yielded strong regression models for all classes, identifying specific molecular biomarkers that are independent from clinical bias. In particular, OA patients and ONA subjects were clearly discriminated ([Fig 3, A](#)). The dispersion along the $t[1]$ dimension seen for OA patients in comparison with ONA subjects could be due to metabolic fluctuations most likely related to the molecular heterogeneity of asthma phenotypes.⁴

According to an operational definition, a phenotype is “a feature, or more usually, a cluster of features that leads to the separation of a specific group at a given time.”² Therefore because the OA metabolic profile from EBC diverges from both the LA and ONA clusters, it can be ascribed to a separate metabolic phenotype. The identification of a specific OA respiratory phenotype fills a relevant gap in the definition of this elusive phenotype. A distinct obesity-related asthma phenotype was thus far suggested only in nonallergic, late-onset (non- T_H2) asthma,^{11,13} which was positively affected by bariatric surgery,¹¹ but no specific biomarkers up to now defined obesity-related asthma in T_H2 and non- T_H2 types.

The metabolic signature we describe for OA can be validly considered specific manifestation of an OA respiratory phenotype

based on both conceptual and experimental evidence. A phenotype might be defined by 1 or more domains (clinical characteristics and specific cellular patterns in biological samples)² in which metabolic profiles and the metabolic pathways in which they participate vary according to the biological matrix involved. Along these lines, the EBC metabotype of LA patients we previously described as discriminative versus nonasthmatic control subjects²³ recalls the obese urinary metabotype²⁸ because both matrices identify obese subjects based on metabolites specific to each compartment. This is in line with other studies of NMR-based metabolic profiling of obesity conducted in animal models in which different biological samples (eg, serum, urine, and others) consistently detected distinct biomarker signatures in obese mice with respect to lean control animals,²⁹ as well as in patient-based studies on obesity in which differences in sex, pubertal development, and physical activity were derived from plasma and urine metabolomes in overweight adolescents.³⁰

The clear separation observed in the 3 metabolic profiles strongly supports the notion that they represent pathophysiologic processes somewhat specific for each condition. Consequently, the pathways involved in OA patients could indicate biochemical mechanisms approachable by specific pharmacologic treatments. Analysis of metabolites defining the OA phenotype uncovered molecular species not previously associated directly with OA. Pathway topology analysis uncovered methane, pyruvate, and glyoxylate and dicarboxylate metabolisms as the most probable pathways involved in the class separation. An association between methane metabolism and obesity has been reported,³¹ and in human subjects methane metabolism is associated with greater BMI and percentage body fat.³² Furthermore, subjects with increased methane metabolism might present with alteration in glucose metabolism and altered glycemic control.³³

Surprisingly, we detected a relevant increase of glucose levels in EBC when comparing OA patients and ONA subjects ([Fig 3, B](#)) and all classes ([Fig 5, B](#)), although all the enrolled subjects

were normoglycemic. Such a glucose increase is not directly linked to obesity itself because the obesity-asthma comorbidity only presents a relatively small increase compared with asthma alone (Fig 4, B). Glucose concentrations in the breath can increase because of pathologic or physiologic processes, as observed in the EBC of patients with cystic fibrosis and might predispose to pulmonary exacerbations³⁴ and infection.³⁵ Our results indicate that in normoglycemic obese subjects the presence of asthma increases the EBC glucose level with respect to lean subjects and markedly with respect to ONA subjects. Furthermore, for the first time, we show that detection of breath glucose can be easily obtained by using NMR-based metabolomics of EBC samples.

The OA class in both S-plots shows a reduction of methanol, formate, and acetate levels, which can all be involved in the methane metabolism.³⁶ From the peak intensity, for the OA class in the ONA-OA model, the reduction of formate, methanol, and acetate concentrations is smaller (Fig 3, B) if compared with the OA-LA S-plot (Fig 4, B), in which they present a larger decrement. Because methane metabolism is an important pathway involved in the production of cellular energy, our findings suggest that the energy requirement is different in the 2 conditions. The progression from ONA to OA seems to require lower energy, consuming a relatively low amount of formate, methanol, and acetate. On the contrary, from LA to OA, the obesity-asthma comorbidity involves additional energy, consuming more substrates because of the upregulation of methane metabolism.

The methane and pyruvate pathways are strictly linked in the requirement of energy. The pyruvate pathway is the sum of all biochemical reactions involving pyruvate and is at the intersection of pathways important for glucose and energy homeostasis for its conversion into carbohydrates, fatty acids, and energy through acetyl-CoA and ethanol. Pyruvate is the byproduct of glucose, which, through the Krebs cycle, forms 2 molecules of pyruvic acid, which is reported to be a potent scavenger of free radicals showing potent antioxidant and anti-inflammatory actions.³⁷ Pyruvate keeps a high ATP concentration in the liver to metabolize fat at the highest possible rate.³⁸ Putative involvement of methane and pyruvate has not been reported thus far in studies on asthma pathophysiology.

The glyoxylate and dicarboxylate cycle uses acetate for growth and energy and results in the formation of malate from 2 molecules of acetate. Both glucose and fatty acids can be converted into acetate for use in the glyoxylate cycle, which is particularly important for use of fatty acids as substrates for growth.³⁹ The alteration of glyoxylate and dicarboxylate metabolism in aged human female subjects has been related to mitochondrial dysfunction that would result in decreased ability to detoxify reactive oxygen species (ROS).⁴⁰

Many of the altered metabolites are also suggestive of inflammation. Methanol and formate are downgrading products of formaldehyde, which has been reported to exacerbate airways inflammation in A549 alveolar and BEAS-2B bronchial cell lines⁴¹ and in male Wistar rats.⁴² Therefore reduced methanol and formate levels in OA patients with respect to LA patients (Fig 4, B) and, to a lesser extent, with respect to ONA subjects (Fig 3, B) might indicate an endogenous mechanism for limiting pulmonary inflammation. In line with this, an increase in levels of acetoin, which is involved in pulmonary redox

cycling, might generate toxic injurious ROS,⁴³ therefore harming lung tissue.

Acetoin, which is detected in the breath of patients with cystic fibrosis, causes direct structural damage and likely interacts with the host immune system.⁴⁴ Therefore acetoin increases linked to obesity and augmented in the presence of obesity and asthma might be explained by the ROS-induced inflammation, and its increase in OA patients compared with LA patients (Fig 4, B) and ONA subjects (Fig 3, B) might be associated with a different airway inflammation pattern in patients with these disorders.

Short-chain fatty acids (SCFAs; acetate, propionate, butyrate, and *n*-valerate) regulate several leukocyte functions linked to the production of cytokines, eicosanoids, and chemokines and seem to affect leukocyte migration to the foci of inflammation.⁴⁵ Because they are also involved in the energy requirement, their decrease might reflect the tight interplay between inflammation and energy production.

At physiologic concentrations, ethanol is oxidized by alcohol dehydrogenase, generating an increased NADH/NAD⁺ ratio in the liver, which in turn inhibits fatty acid oxidation.⁴⁶ Such inhibition increases intracellular ROS. On the contrary, mitochondrial fatty acid oxidation might provide NADPH to regenerate the glutathione antioxidant system for defense against oxidative stress and prevent ATP loss and cell death.⁴⁷ Because of the inflammation caused by obese asthma, it could be hypothesized that the decrease in ethanol might enhance the defense mechanisms against oxidative stress by favoring the parallel increase in the NADH/NAD⁺ ratio and fatty acid oxidation for NADPH production. Lastly, acetone was detected in the bronchoalveolar lavage fluid of pediatric patients with cystic fibrosis, with varying levels of inflammation.⁴⁸

Kim et al⁴⁹ reported that mice fed a 14-week high-fat diet had obesity and also airway hyperreactivity, a characteristic of asthma, which can be associated with activation of the NLRP3 inflammasome, IL-1 β production, and increased numbers of IL-17A-producing type 3 innate lymphoid cells in the lungs. Interestingly, fatty acid palmitate, cholesterol crystals, and ceramide (generated from fatty acids) are all able to activate NLRP3.⁴⁹ Palmitic acid (its anion palmitate is the observed form at physiological pH) is the most common saturated fatty acid found in animals. Excess carbohydrates in the body are converted to palmitic acid, which is also the first fatty acid produced during fatty acid synthesis and the precursor to longer fatty acids.

Some of our data align with this experimental evidence. In OA patients saturated fatty acid concentrations decrease with respect to ONA subjects and LA patients, allowing speculation that fatty acids could be used for activation of IL-17A and the NLRP3 inflammasome pathway in the OA group. Also, the acetate decrease could be involved because, as shown in the mevalonate pathway, cholesterol is formed from acetate units. Therefore acetate reduction in OA patients could indicate active cholesterol synthesis, leading to activation of the NLRP3 inflammasome by cholesterol crystals.

Our data also agree with evidence regarding the potential positive effects of dietary fiber on allergic lung inflammation.⁵⁰ Mice fed a high-fiber diet and exposed to house dust mite extract had less severe allergic airway inflammation and showed reduced airway hyperreactivity compared with mice on a control diet. An

increase of SCFA levels in the serum was seen in mice on a high-fiber diet. Treatment of mice with propionate reduced the lung inflammatory response after house dust mite exposure, and this effect was dependent on the SCFA receptor G protein-coupled receptor 41 (GPR41; also known as FFAR3). Acetate, propionate, and butyrate are the most extensively described SCFAs, and although they are not present in lung tissue, we hypothesize that the decrease in SCFA levels we observed for OA might exert a protective effect on airway inflammation. Because the reduced inflammation in the propionate-treated mice was primarily due to reduced eosinophilia, which is suggestive of an impaired T_H2 cell response,⁵⁰ it remains an open question whether and how SCFAs act on non- T_H2 asthma. Interestingly, a general anti-inflammatory action for propionate has been put forward.^{45,51}

Because the subjects involved in the study were recruited through the hospital's surgery department from obese patients scheduled for bariatric surgery, our selection of patients with mild-to-moderate asthma and obesity (BMI >30 kg/m²) was due to requirements of the surgical protocol, which excludes patients with severe asthma and severe comorbidities. Therefore the presence of the respiratory metabolic phenotype awaits validation in OA patients with different degrees of asthma severity. Recently, a metabolomic analysis of plasma found that children with severe asthma are differentiated from patients with mild-to-moderate asthma.⁵² It is unclear whether similar differences can also be detected in airway samples, but our analysis was not affected by the presence of a small number of patients with different GINA grades within the considered mild-to-moderate bracket (Table I). This could be in line with the suggestion that phenotypes based on biomarkers appear to be more stable in patients with mild-to-moderate asthma with respect to those with severe asthma.⁵³

Lack of correlation of our data with clinical parameters somehow limits the interpretation of the data. This limitation is likely the expression of the "low resolution" that a single biofluid (like EBC) can generate in the understanding of a complex system, which is globally represented by the clinical parameters. The current undertaking in our group is the coupling of mass spectrometry and NMR techniques to study the metabolic profile of multiple biofluids (serum, urine, EBC, and saliva) in obese subjects. By integrating the metabolomic profiles of different biofluids, we aim at better defining the pathways involved in discrimination among the 3 groups. Moreover, comparison of the metabolomic data with more mechanistic parameters (eg, cellular and mediator patterns) could shed additional light on the cellular sources and disease-specific mechanisms that originated the metabolites and the pathogenetic role of the metabolic pathways in which they participate, clarifying partially the relationship with clinical parameters.

Considering these limitations, our findings, obtained by using an unbiased methodology, unequivocally indicate that patients with obesity and asthma diverge qualitatively from patients affected by obesity or asthma alone by virtue of a highly specific respiratory metabolic profile. This novel characteristic adds strong experimental evidence to the definition of a specific asthma phenotype occurring in adult OA patients.

We thank all patients for their participation in the study.

Key messages

- OA patients have a specific respiratory metabolic phenotype ("metabotype") that diverges from that of patients affected by obesity or asthma alone.
- The pathogenesis of obese asthma is differently regulated with respect to obesity or asthma.
- The specific OA metabotype could help in the initial patient stratification for a more personalized pharmacologic treatment with enhanced effectiveness.

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METHODS

Study subjects

Height, weight, and waist and hip circumferences were obtained according to the National Health and Nutrition Examination Survey Web site (<http://www.cdc.gov/nchs/nhanes.htm>). Estimation of body composition was achieved by using the Tanita-BC-418 8-electrode bioelectrical impedance analysis device (Tanita), for which participants were asked to follow all recommended guidelines, according to the manufacturer's instructions.

The average age of the enrolled and validation subjects were, respectively, as follows: 38.3 years (range, 30.1–50.6 years) and 36.4 years (range, 29.9–47.3 years) for ONA subjects; 36.7 years (range, 29.3–53.4 years) and 38.3 years (range, 30.3–50.1 years) for LA patients; and 39.0 years (range, 31.4–48.2 years) and 40.4 years (range, 31.8–49.9 years) for OA patients. The average BMIs of the enrolled and validation subjects were, respectively, as follows: 46.1 kg/m² (range, 33.7–49.4 kg/m²) and 44.7 kg/m² (range, 34.2–49.7 kg/m²) for ONA subjects; 21.6 kg/m² (range, 18.2–22.8 kg/m²) and 20.0 kg/m² (range, 17.0–24.2 kg/m²) for LA patients; and 45.3 kg/m² (range, 34.2–50.2 kg/m²) and 46.2 kg/m² (range 34.2–50.2 kg/m²) for OA patients (Table I).

Lung function and methacholine challenge tests

Lung function measurements were done with a water-sealed spirometer (VMax Encore, CareFusion). The software automatically chose the best of 3 measurements. FEV₁, FVC, and FEV₁/FVC ratio percentages were selected as the parameters of interest.

A 2-minute methacholine provocation test was performed by using a sidestream nebulizer (Jaeger APS pro, CareFusion Germany) and doubling concentrations of methacholine bromide (Lofarma, Milan, Italy). FEV₁ was used to quantify changes in airway caliber. The bronchial response was expressed as the provocative concentration of methacholine causing a 20% decrease in FEV₁ (PC₂₀, in milligrams or milliliter) and was calculated by means of linear interpolation of the log dose-response curve. Bronchial hyper-responsiveness was defined as provocative concentration of methacholine causing a 20% decrease in FEV₁ of 16 mg/mL or less.

Power analysis

Because there are no standardized methods for evaluating the power of the analysis for projection methods, such as OPLS analysis, we consider our study a pilot study for which no *a priori* power analysis was possible. We used our results to backward evaluate the power of our analysis because biomarkers and their concentration changes that could determine class separation were unknown before analysis. By varying the parameters 1- α from 95% to 99.9% and 1- β from 80% to 99.9% and using the accuracy percentages obtained for our validation tests for unexposed and exposed subjects (see the Results section), for a 1- α value of 95% and a 1- β value of 80%, we obtained a number of subjects corresponding to 18 $\pm$ 2 healthy obese subjects, 19 $\pm$ 3 LA patients, and 20 $\pm$ 2 obese patients with mild-to-moderate asthma, whereas for a 1- α value of 99.9% and a 1- β value of 99.9%, we obtained 19 $\pm$ 3 healthy obese subjects and 20 $\pm$ 3 patients for both classes of asthma. In this study we used a number of subjects for each class that complies with or is larger than numbers suggested by backward analysis. Typically, the 1- α value is 95% and the 1- β value is at least equal to 80%, and a value of 99.9% represents an extreme requirement.

EBC sampling

EBC samples were collected by using a Jaeger EcoScreen condenser (CareFusion Germany) and randomizing the sequential order of the subject collection.^{E1,E2} Condensing temperature was measured before and after each sample collection, with $-27.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ as the average value. Briefly, subjects were asked to breathe tidally through a mouthpiece into a 2-way nonbreathing valve, which also served as a saliva trap, for 15 minutes while wearing a nose clip. All the subjects collected their EBC in the same room in 1 week. EBC samples were centrifuged immediately after collection and transferred into 5-mL glass vials. Before sealing, volatile substances were

removed from samples by using a gentle nitrogen gas flow for 3 minutes. Sealed samples were then frozen in liquid nitrogen to immediately “quench” metabolism and preserve the metabolite concentrations. The samples were stored at -80°C until NMR analysis, which was performed within 1 week from sampling time.

Salivary contamination of the samples was tested by measuring their α -amylase activity. The room temperature remained constant ($24^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$) throughout the sampling period. Possible air contaminants in the collecting room were monitored with a dedicated sampling pump for air monitoring (Zambelli Ego Plus TT; Zambelli, Milan, Italy), working at a flow rate of 8 L/min and tidal volume (500 mL) into the condenser, so as to simulate human breath.^{E3} The pump was connected to the condenser outlet for 15 minutes, and special filters (3M Particulate Filters P100; 3M Italia, Milan, Italy; tested against particles approximately 0.3 μm in size) for respiratory protection were applied to the 1-way valve of the mouthpiece condenser used for the whole set of experiments. NMR spectra of condensed room air from the collecting device were devoid of signals, confirming the absence of air pollutants (data not shown).

To reduce the risk of contamination by inhaling hospital air, subjects were sampled after a 30-minute rest in the greenhouse of the Department of Respiratory Medicine, which was shown to be contaminant free as described above for the collecting room.

NMR sample preparation

EBC samples were rapidly defrosted. To provide a field frequency lock, 70 μL of a ²H₂O solution (containing 0.1 mmol/L sodium TSP as a chemical shift reference for ¹H spectra and sodium azide, a bacteriostatic agent at 3 mmol/L) was added to 630 μL of EBC, reaching 700 μL of total volume.

NMR spectroscopy measurements

All spectra were recorded on a 600-MHz Bruker Avance-III spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a CryoProbe. 1D ¹H-NMR spectra were collected at 300 K with the excitation sculpting pulse sequence to suppress the water resonance.^{E4} We used a double-pulsed field gradient echo, with a soft square pulse of 4 ms at the water resonance frequency and gradient pulses of 1 ms each in duration, adding 128 transients of 64k complex points, with an acquisition time of 4 s/transient. Time-domain data were all zero-filled to 128k complex points, and before Fourier transformation, an exponential multiplication of 0.6 Hz was applied. 2D clean TOCSY spectra were recorded by using a standard pulse sequence^{E5} and incorporating the excitation sculpting sequence for water suppression. In general, 320 equally spaced evolution time period t_1 values were acquired, averaging 4 transients of 2048 points. Time-domain data matrices were all zero-filled to 4096 points in both dimensions, and before Fourier transformation, a Lorentz-to-Gauss window with different parameters was applied for both t_1 and t_2 dimensions for all the experiments. The spectral positions of the lines (“resonances”) in both homonuclear 1D and 2D spectra were referred to the spectral position of the signal originating from 0.10 mmol/L TSP, which was assumed to resonate at a δ value of 0.00 ppm.

For the natural abundance of 2D ¹H-¹³C HSQC spectra, we used an echo-antiecho phase sensitive pulse sequence by using adiabatic pulses for decoupling.^{E6} One hundred twenty-eight equally spaced evolution-time period t_1 values were acquired, averaging 48 transients of 2048 points and using GARP4 for decoupling. The final data matrix was zero-filled to 4096 in both dimensions and apodized before Fourier transformation by a shifted cosine window function in t_2 and in t_1 . Linear prediction was also applied to extend the data to twice their length in t_1 . The spectral positions of the “resonances” were referred to the lactate signal (βCH_3), which was assumed to resonate at 1.33 ppm for ¹H and 20.76 ppm for ¹³C.

Spectral and statistical analysis

Before analysis, within-day, between-day, and technical repeatabilities and detection limits were assessed according to published protocols.^{E2,E7,E8} Regarding within-day repeatability, the integration of selected spectral regions

showed that, according to the Bland-Altman test, all values fell within 2 SDs (mean, 0.86; ie, between -0.41 and 0.45), which indicates good repeatability. The intraclass correlation coefficient was used to assess between-day and technical repeatabilities, obtaining 0.99 for both. The measured detection limit was $0.10 \pm 0.04 \mu\text{mol/L}$ for phenylalanine, which is the metabolite with the lowest detectable concentration.

Discriminating elements in NMR spectra are better appreciated by using multivariate analysis, which carefully identifies hidden phenomena and trends in ensembles of spectra. Phased- and baseline-corrected ^1H -NMR spectra were automatically data reduced to 390 integral segments ("buckets"), each of 0.02 ppm, by using the AMIX 3.9 software package (Bruker Biospin GmbH) between 0.70 and 8.50 ppm. The residual water resonance region (5.20-4.46 ppm) was excluded, and each integrated region was normalized to the total spectrum area to avoid possible signal variation because of dilution of EBC samples, which could result in artificially increasing of the signals.

To discriminate different EBC samples through NMR spectra, we carried out a multivariate statistical data analysis using projection methods, as implemented in the SIMCA-P+ 13 package (Soft Independent Modeling of Class Analogy; Umetrics, Umeå, Sweden). Because EBC spectra present signals of different intensities, the data were preprocessed with Pareto scaling to make comparable the contribution of resonances in EBC spectra while softening the noise effect. Each region was scaled to $(1/S_k)^{1/2}$, where S_k is the standard deviation for the variable k , increasing the contribution of lower concentration metabolites to the models generated compared with models where no scaling is used. The model quality was evaluated by using the goodness-of-fit parameter (R^2) and the goodness-of-prediction parameter (Q^2). For them, acceptable values must be 0.5 or greater, with $|R^2 - Q^2| < 0.2$ -0.3.^{E9}

Principal component analysis was first applied to detect EBC metabolite trends and clusterings in an unsupervised (ie, no prior group knowledge is used in the calculation) manner. However, to better identify clusterings, we tested both OPLS and OSC routine^{E10} together with the PLS-DA to verify data fitting and possible data overfitting. We applied the OSC filter to spectral data to test whether it could remove X variables unrelated (orthogonal) to Y response matrix (class belonging) to obtain a clear separation of sample classes. Indeed, unlinked variables or systematic noise can hamper multivariate modeling and cause imprecise predictions for new samples. In our case we considered a 2-component OSC model with the corresponding 36.55% of remaining sum of squares in the X-block, reaching an angle of 89.97 degree between t and Y. A 2-component PLS-DA model was subsequently generated with an R^2 value of 0.932 and a Q^2 value of 0.805; the 7-fold cross-validation and permutation tests (800 repeats) with respect to lean class yielded an R^2 value of 0.591 and a Q^2 value of -0.343 as intercept values (which suggests a nonoverfitting model), whereas the cross-validated-ANOVA test produced a P value of 9×10^{-6} . For the orthogonal projections to latent structures-discriminant analysis (OPLS-DA) model, we obtained a P value of .001. Notwithstanding the difference in P values, because the OSC filter is prone to overfitting and considering the limited number of samples, we finally applied OPLS-DA to the original data set (the unfiltered one) as discriminant analysis, with an internal orthogonal filter applied to remove noncorrelated variation and generate a single predictive X block that is correlated with the Y response. The obtained OPLS model turned out to be improved in terms of both predictive and interpretive ability.

The statistical models we presented in the article were obtained by applying multivariate statistical data analysis, the strength of which is to transform the original variables to create and use a reduced set of variables that are much easier to analyze and interpret. The collected data set under investigation is

represented by an X matrix containing N observations (samples) and K independent variables (NMR signals), whereas class belonging of each sample is organized in a discriminant matrix Y. To extract meaningful information from our samples, we applied OPLS-DA, a regression model that searches for a set of components (called latent vectors) that performs a simultaneous decomposition of X and Y with the constraint that these components explain as much as possible of the covariance between X and Y, so that the source of variation in X (independent variables or predictors) will be in principle used to explain and predict Y (dependent variables or responses). Moreover, an internal filter (called orthogonal) is able to reduce the effects of the structured noise in data modelling so that the X-block is decomposed into 2 different parts: one related to the predictive latent components useful to explain the response and the other associated to the nonpredictive latent components orthogonal to the response. Hence the regression decomposition of our models results in the following equation:

$$X = T_p P_p^T + T_o P_o^T + E,$$

where T_p denotes the predictive score matrix for X, P_p is the predictive loading matrix for X, T_o denotes the corresponding Y-orthogonal score matrix, P_o represents the loading matrix of Y-orthogonal components, and E denotes the residual matrix of X. Because the predictive variation of Y in X is modeled by the predictive components, we analyze the predictive block X_p as follows:

$$X_p = X - T_o P_o^T.$$

Therefore our models are represented by projection of the acquired data onto t_p predictive scores and p_p predictive loadings that decompose the predictive block, thus improving model interpretability in correlation with sample classification according to the clinical condition under investigation.

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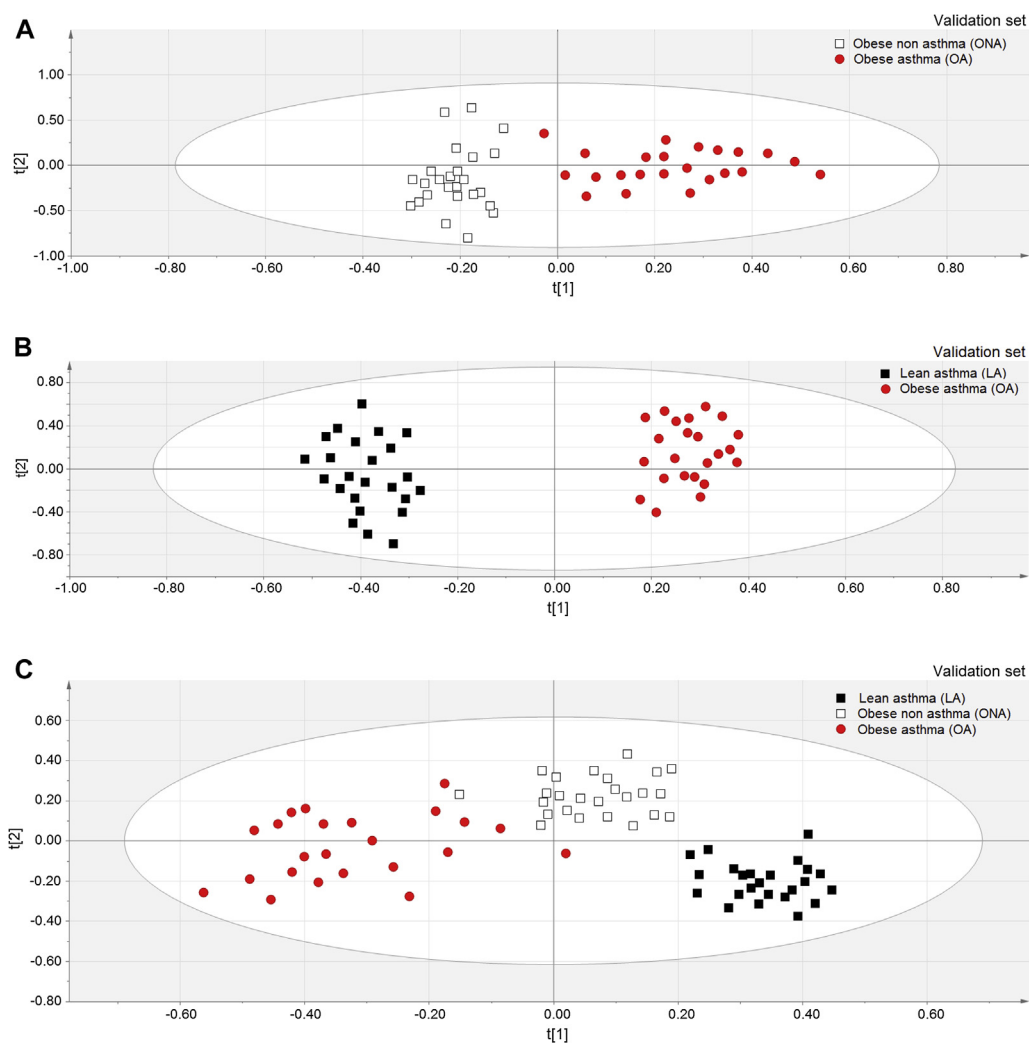


FIG E1. OPLS-DA of EBC samples from the validation set. **A**, Score plot showing the degree of separation of the model between ONA subjects (white squares) and OA patients (red circles). **B**, Score plot showing the model between LA patients (black squares) and OA patients (red circles). **C**, Score plot of the model between LA patients (black squares), ONA subjects (white squares), and OA patients (red circles). The labels $t[1]$ and $t[2]$ along the axes represent the scores (the first 2 partial least-squares components) of the model, which are sufficient to build a satisfactory classification model.

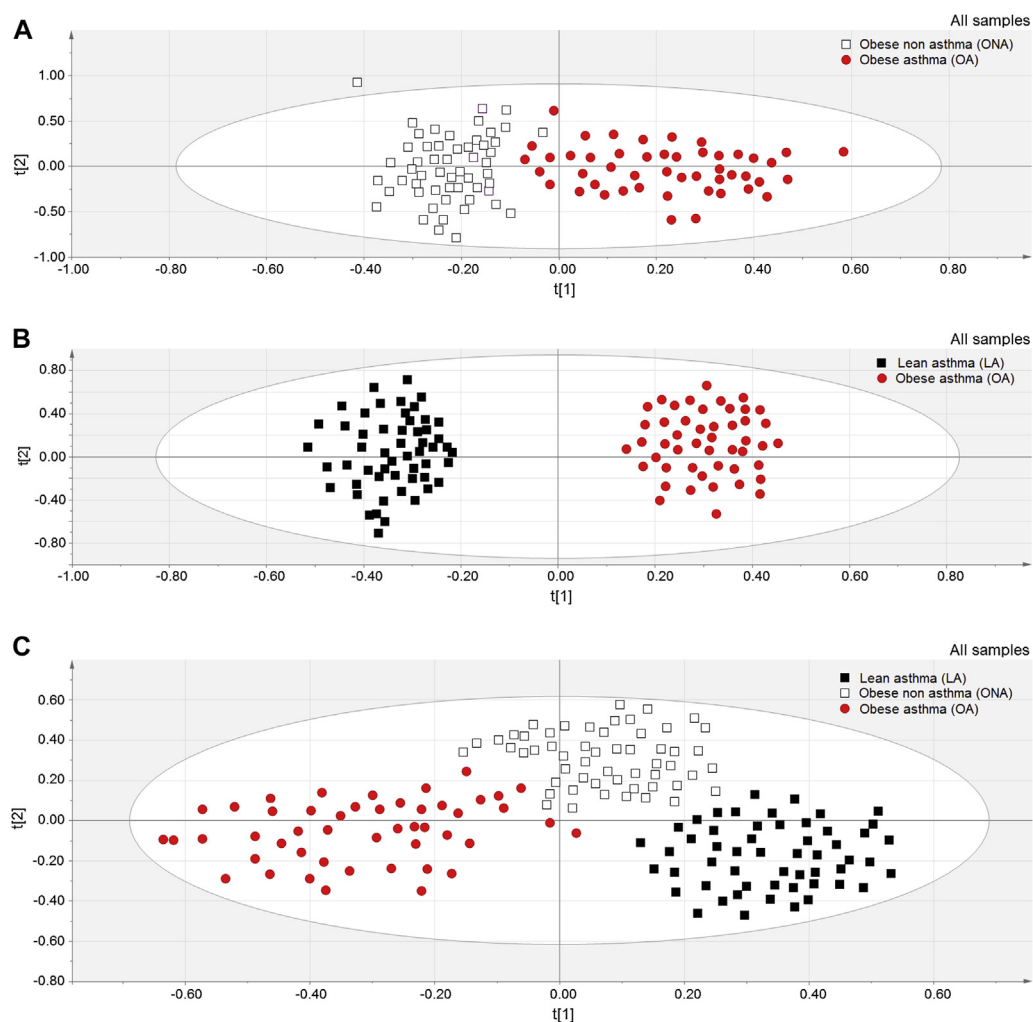


FIG E2. OPLS-DA of EBC samples from all (original and validation) sample sets. **A**, Score plot showing the degree of separation of the model between ONA subjects (*white squares*) and OA patients (*red circles*). **B**, Score plot showing the model between LA patients (*black squares*) and OA patients (*red circles*). **C**, Score plot of the model between LA patients (*black squares*), ONA subjects (*white squares*), and OA patients (*red circles*). The labels $t[1]$ and $t[2]$ along the axes represent the scores (the first 2 partial least-squares components) of the model, which are sufficient to build a satisfactory classification model.

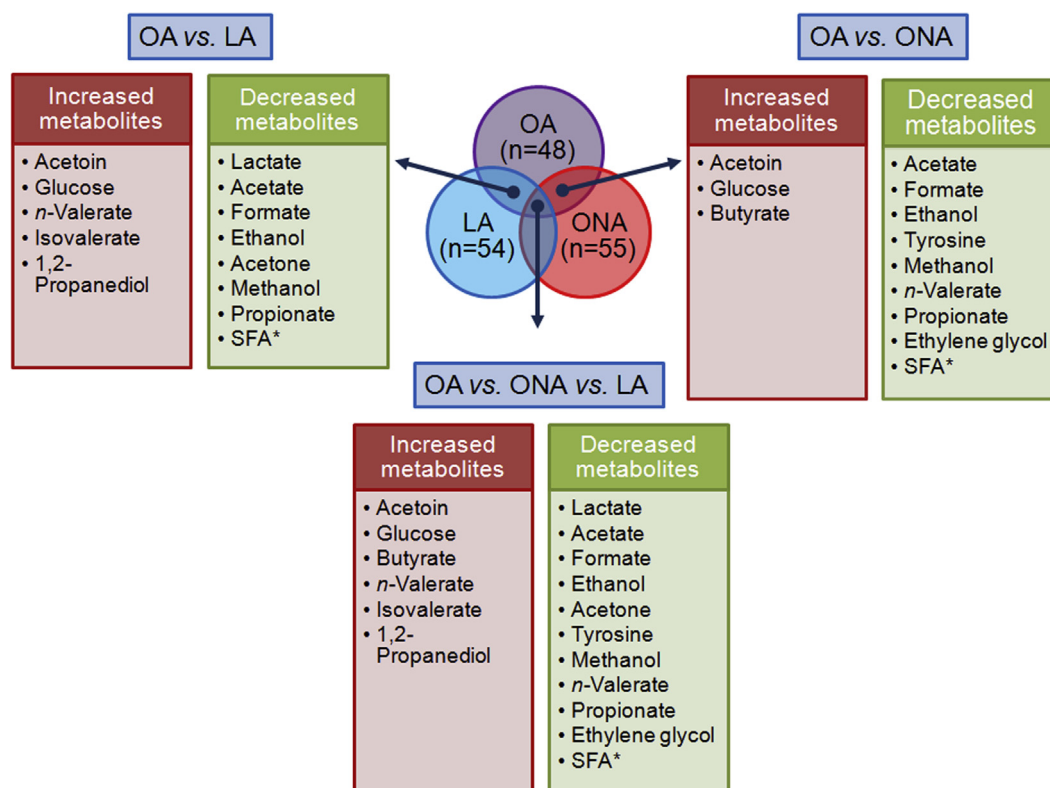


FIG E3. Discriminating metabolites for the all-subject analysis (research set plus validation set). Metabolites are derived from the loading plots associated to the scores plots reported in Figs 3, B; 4, B; and 5, B, as well as the loading plots (not shown) associated to the scores plots of the validation datasets reported in Figs E1 and E2. Metabolites responsible for between-class differences (OA patients vs ONA subjects vs LA patients) correspond to those recognized in the 2-class comparisons shown above. *SFA, Saturated fatty acids.