

Carraro Silvia (Orcid ID: 0000-0003-2284-1225)
 Licari Amelia (Orcid ID: 0000-0002-1773-6482)

METABOLOMICS TO IDENTIFY OMALIZUMAB RESPONDERS AMONG CHILDREN WITH SEVERE ASTHMA: A PROSPECTIVE STUDY

Silvia Carraro¹, Emanuela di Palmo², Amelia Licari³, Simona Barni⁴, Valeria Caldarelli⁵,
 Giovanna De Castro⁶, Antonio Di Marco⁷, Grazia Fenu⁸, Giuseppe Giordano^{9,10}, Enrico
 Lombardi⁸, Paola Pirillo^{9,10}, Matteo Stocchero^{9,10}, Alessandro Volpini¹¹, Stefania Zanconato¹,
 Franca Rusconi¹² and the Italian Paediatric Severe Asthma Network (IPSAN)

¹Unit of Pediatric Allergy and Respiratory Medicine, Women's and Children's Health Department,
 University of Padova

²Pediatric Unit – IRCCS Azienda Ospedaliero-Universitaria di Bologna

³ Pediatric Clinic, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Pavia

⁴Allergy Unit, Meyer Paediatric University Hospital, Florence

⁵Pediatric Unit, Department of Obstetrics, Gynecology and Pediatrics, Azienda USL - IRCCS, Reggio
 Emilia

⁶Pediatric Allergology Unit, Maternal and Child Department and Urological Science, Umberto I
 Hospital, Sapienza University, Rome

⁷Pediatric Pulmonology & Respiratory Intermediate Care Unit, Sleep and long term Ventilation Unit,
 Academic Department of Pediatrics (DPUO), Bambino Gesù Children's Hospital, IRCCS, Rome

⁸Pulmonary Unit, Meyer Paediatric University Hospital, Florence

⁹Mass Spectrometry and Metabolomic Laboratory, Women's and Children's Health Department,
 University of Padova, Padova

¹⁰Fondazione Istituto di Ricerca Pediatrica (IRP), Padova

¹¹Pediatric Unit, Department of Mother and Child Health, Salesi Children's Hospital, Ancona

¹²Department of Mother and Child Health, Azienda USL Toscana Nord Ovest, Pisa, Italy.

Authors' contribution

SC, SZ, FR conceived the study; EP, AL, SB, VC, GDC, AdM, GF, EL, AV recruited and followed the
 patients; SC, EdP, FR coordinated the centres; PP, GG analysed the urine samples; PP, MS performed
 the statistical analysis, SC wrote the first draft; AL, SZ, FR contributed to manuscript writing.

Corresponding Author

Silvia Carraro, MD
 Unit of Pediatric Allergy and Respiratory Medicine
 Women's and Children's Health Department
 University of Padova
 Padova, Italy
 Email: silvia.carraro@unipd.it

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To the Editor.

In this study, we aimed to assess the potential value of baseline urinary metabolomic profiles in predicting the response to omalizumab in children with severe asthma. Although most children show a good response to this drug¹ 10-15% are partial or non-responders.² Identifying the patients most likely to benefit from omalizumab is crucial to personalise the treatment and optimise cost-effectiveness. So far, clinical or biochemical predictors of response to omalizumab have not been definitively recognised. Metabolomic analysis, using an untargeted approach, has the potential to enable the identification of metabolic features associated with relevant clinical outcomes.³

In this multicentre prospective study (Ethics Committee approval 4329/AO/17), after obtaining informed consent, we consecutively recruited patients 6-17 years old with severe asthma participating in a national database of the Italian Society for Paediatric Respiratory Diseases, who were candidates for omalizumab. Children had been treated for at least four months with a daily ICS dose ≥ 500 mcg of fluticasone propionate or equivalent. A urine sample was collected before starting omalizumab. After 52 weeks of follow-up, or sooner if omalizumab was stopped before, children were classified as responders or not, using a multidimensional approach.⁴ We considered the variation from baseline of GINA score (which evaluates asthma control in the last four weeks) and CASI⁵ score (which evaluates control in the last two weeks together with lung function, exacerbations and treatment). We defined as responders children with at least a 1-point reduction in CASI score⁶ and controlled or partially controlled according to GINA. Since QoL is significantly affected in severe asthma,⁷ in case of inconsistency, we considered as responders those with an increase of at least 0.5 points⁸ in Pediatric Asthma Quality of Life Questionnaire (PAQLQ) score.⁹

Urine analysis was performed through high-resolution mass-spectrometry (Q-ToF Synapt G2, Waters, Milford, USA) interfaced with the chromatography Acquity UPLC using the reverse phase column HSS T3 (Waters, Milford, USA).

Children's baseline characteristics were compared by t-test, Mann-Whitney test, and Fisher's exact test; in case of differences in their distribution, sub-sampling was performed to match responders and non-responders (a standard procedure in metabolomic studies to avoid false discoveries due to experimental design bias).

For metabolomic analysis a two-group comparison and a one-class modelling approach were applied. The former assumes that responders and non-responders belong to two different groups, each metabolically well-defined, and it was based on univariate methods (Mood's median test controlling the false discovery rate by Storey method, adjusted $p < 0.15$). The one-class approach assumes that responders belong to a metabolically homogeneous group while non-responders are scattered around it, and it was based on multivariate (Principal Component Analysis) and univariate methods for outlier detection. With the univariate approach the distribution of each metabolic variable in responders was modelled by kernel density estimation, the probability of each non-responder being an outlier for that variable was estimated and false discovery rate was controlled by Storey method (adjusted $p < 0.15$).

The relevant variables were annotated searching our database of commercial standards, METLIN database and Human Metabolome Database.

52 children were included in the analysis (10 non-responders) (table 1).

Metabolic features were differently expressed in responders and non-responders.

The two-class comparison approach highlighted 17 discriminating features (figure 1); among these, six metabolites were identified (putative biomarkers), four higher in responders and two in non-responders (table 1).

Biomarkers increased in responders were dipeptides and amino acids, in keeping with previous studies that suggest an altered amino acid metabolism in asthma.¹⁰ The better characterised were the histamine precursor L-Histidine, a possible marker of an altered histamine pathway, and L-Kynurenine.

On the other hand, uric acid, a metabolite with a possible role in innate and adaptive T2 response amplification,¹¹ was a putative biomarker in non-responders.

Moreover, the one-class modelling approach highlighted 100 features differently expressed in at least three non-responders with respect to responders (annotated metabolites in table S1) and the Q-chart built by PCA was promising for non-responder detection (figure S1 in supplementary).

Details of methods and results are reported in supplementary.

In conclusion, we found that children with severe asthma responding to omalizumab showed a different metabolomic urinary profile at baseline compared to non-responders. A metabotype enriched in amino acids was associated with a good response to omalizumab, while the uric acid metabolic pathway was involved in non-responders.

For the first time, this study paves the way to a possible a-priori identification of children who are most likely to benefit from omalizumab based on their metabolic arrangement. Further studies, also based on targeted approaches, may expand these results.

Silvia Carraro¹,

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⁵Pediatric Unit, Department of Obstetrics, Gynecology and Pediatrics, Azienda USL - IRCCS, Reggio Emilia.

⁶Pediatric Allergology Unit, Maternal and Child Department and Urological Science, Umberto I Hospital, Sapienza University, Rome.

⁷Pediatric Pulmonology & Respiratory Intermediate Care Unit, Sleep and long term Ventilation Unit, Academic Department of Pediatrics (DPUO), Bambino Gesù Children's Hospital, IRCCS, Rome.

⁸Pulmonary Unit, Meyer Paediatric University Hospital, Florence.

⁹Mass Spectrometry and Metabolomic Laboratory, Women's and Children's Health Department, University of Padova, Padova

¹⁰Fondazione Istituto di Ricerca Pediatrica (IRP), Padova.

¹¹Pediatric Unit, Department of Mother and Child Health, Salesi Children's Hospital, Ancona.

¹²Department of Mother and Child Health, Azienda USL Toscana Nord Ovest, Pisa, Italy.

Conflict of Interest statements

GF reports financial and non-financial support from AstraZeneca, Chiesi, Boehringer Ingelheim, GSK, Novartis, Sanofi, outside this work; AV reports financial or non-financial support from Chiesi, Fidia, Sooft, Momento Medico, Lusofarmaco, outside this work; EL reports financial and non-financial support from AbbVie, Angelini, Boehringer, Chiesi, GlaxoSmithKline, Lusofarmaco, Novartis, Restech, Sanofi, Vertex, outside this work; SZ reports financial support from Sanofi, outside this work; AdM reports financial support from GlaxoSmithKline and Sanofi, outside this work. SC, EdP, FR, SB, PP, GDC, VC, AL, GG, MS, report no conflict of interest for this manuscript.

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Table 1. Children's characteristics, clinical variables and identified metabolites

Baseline characteristics	Responders (n=42)		Non-responders (n=10)	
Males (n, %)	23 (55%)		4 (40%)	
Age (mean, SD)	12.2 (2.7)		10.9 (2.3)	
BMI (mean, SD)	20.7 (4.2)		19.8 (2.9)	
Total IgE (IU/ml) (mean, SD)	701.5 (516.3)		494.1 (472.8)	
Allergic comorbidities^ (n, %)	26 (62%)		5 (50%)	
Sensitised to perennial allergens (n, %)	41 (98%)		8 (80%)	
Parental smoking (n, %)	29 (69%)		7 (70%)	
Parental asthma (n, %)	19 (45%)		4 (40%)	
Clinical variables	Baseline	End of follow-up *	Baseline	End of follow-up *
Number of steroid courses in the previous 12 months (mean, SD)	4.2 (4.2)	0.7 (1.2)	4.5 (3.3)	3.4 (4.0)
LABA (n, %)	40 (95%)	32 (76%)	9 (90%)	9 (90%)
Montelukast (n, %)	29 (69%)	9 (21%)	4 (40%)	4 (40%)
FEV1 (% pred) (mean, SD)	91 (19)	96 (16)	91 (14)	85 (11)
GINA score U/P/C (n)	37/5/0	5/9/28	8/2/0	8/2/0
CASI score (mean, SD)	9.8 (3.8)	4.7 (1.7) [¶]	9.6 (4.1)	8.8 (4.6) [¶]
PAQLQ (mean, SD)	4.7 (1.3)	6.5 (0.5) [¶]	4.7 (1.0)	4.1 (0.7) [¶]
Identified metabolites at baseline	Responders (n=42) Median [IQR]		Non-responders (n=10) Median [IQR]	
L-Histidine (178.0590 m/z; RT 0.523) [ID: POS413]	0.23 [0.20-0.34]		0.13 [0.10-0.19] [#]	
Uric acid (169.0361 m/z; RT 0.827) [ID: POS1370]	0.22 [0.10-0.30]		0.42 [0.35-0.58] [#]	
L-Kynurenine (209.0930m/z; RT 1.885) [ID: POS 2148]	0.05 [0.03-0.13]		0.018 [0.007-0.037] [#]	
3-Dimethylallyl-4-hydroxyphenylpyruvate (249.1139 m/z; RT 5.582) [ID: POS7858]	0.12 [0.10-0.15]		0.18 [0.16-0.22] [‡]	
Aspartylglycosamine (316.1141m/z; RT 0.677) [ID: NEG3634]	0.28 [0.18-0.35]		0.13 [0.07-0.22] [‡]	
Aspartyl-Threonine (215.0661 m/z; RT 0.789) [ID: NEG3420]	0.21 [0.15-0.27]		0.12 [0.02-0.15] [‡]	

Abbreviations: BMI: body mass index; GINA: Global Initiative for Asthma; U: uncontrolled asthma; P: partially controlled asthma; CASI: Composite Asthma Severity Index; RT: retention time; ID: variable identifier coded as [ionization mode][identifier];

^Allergic rhinitis and/or atopic dermatitis and/or food allergy;

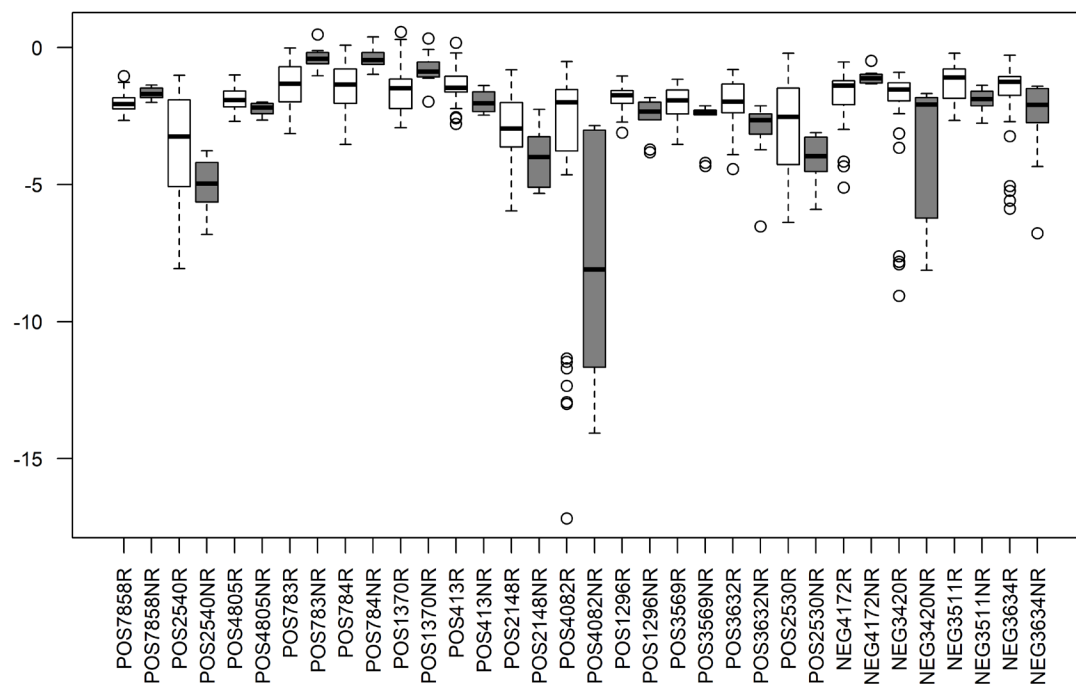
*End of follow-up: evaluation at 52 weeks or when omalizumab was stopped because of poor asthma control (5/10 non responders stopped omalizumab after 4 to 9 months)

† variation from baseline in responders versus non-responders: $p < 0.01$ for CASI; $p < 0.001$ for PAQLQ,

responders versus non-responders $p = 0.01$;

‡ responders versus non-responders $p < 0.001$

Figure 1. Boxplots showing the distributions of the features selected by Mood's test controlling the false discovery rate by Storey method (adjusted- $p < 0.15$); grey bars: non-responders (NR); white bars: responders (R); variables are indicated as [ionization mode][ID][group].



SUPPLEMENTARY MATERIAL

METABOLOMICS TO IDENTIFY OMALIZUMAB RESPONDERS AMONG CHILDREN WITH SEVERE ASTHMA: A PROSPECTIVE STUDY

METHODS

Urine collection and analysis

Urine samples were collected in test tubes pre-washed with methanol to avoid possible contaminations from plastic components and stored at -80°C until analysis.

Urine sample preparation

Urine samples, after being thawed at room temperature, were stirred for 30 s in a vortex mixer, and then centrifuged at 6000 g for 10 min to remove the sediment present in the urine. 100 µL of the supernatant were added to 400 µL of a solution of water with 0.1% formic acid (FA) to obtain a 1:5 dilution. The diluted sample was transferred into two 96 wells plates suitable for the auto-sampler, kept at 5°C.

Chromatographic and mass spectrometry analysis

The metabolic analysis of the collected urine samples was performed using the high resolution mass spectrometer Q-ToF Synapt G2 (Waters, Milford, MA USA) interfaced with the chromatographic system Acquity UPLC (Waters, Milford, MA USA), characterized by high chromatographic resolution, short analytical time and enhanced sensitivity. Solvents and reactants with LC-MS ULTRA grade (Sigma St. Luis, MO, USA) and water type MilliQ with 1 grade of purity were used for the analysis.

The chromatographic analysis was performed through the reverse phase column HSS T3 1.8 µm 2.1 mm and 100 mm (Acquity HSS T3, Waters co., Milford, MA USA) at 50°C. The solvents used for eluent A were H₂O and 0.1% of FA, and methanol:acetonitrile 90:10 and 0.1% FA for eluent B. Percentage of eluent B varied through time in the current way: at time 0 min 5%, at 3.5 min 30%, at 6.5 min 95%, at 11 min 5%, with a constant flow of 500 µL/min. The mass spectrometry analysis was conducted with an Electrospray source (ESI) in both positive (ESI+) and negative (ESI-) ionization mode. The setting of the ESI was arranged at a capillary voltage of 3 KV in the positive polarity and 1.5 KV in the negative one, the source temperature being of 110°C and the desolvation temperature of 350°C with nitrogen gas flow at 600 L/h. The mass scan was set in a range between 20 and 1200 amu in 0.3 s. Data were collected in continuum (profile) mode, in a scanning range of 20-1200 m/z, with acquisition rate of 0.3 s, and with lock mass scans collected every 10 s and averaged over 3 scans for mass correction.

We collected the data also in MS^E scan mode. This last is a non-selective fragmentation technique based on the passage of ionized molecules through the collision cell at high and low collision energy to obtain structural information of the molecules.

To verify the analytical reproducibility during the chromatographic analysis, quality controlsamples (QCs) obtained diluting a mix of 50 µL of each urine samples were injected every 11 injections. Specifically, QCs with dilution factors 1:2, 1:3, 1:5 and 1:7 were employed to better control the analytical session and to calibrate the urine samples in the pre-processing step. A standard mix of known compounds (Sulfadimethoxine, Cholic acid, Leucine-Enkephalin, Erythromycin, Hippuric acid, Acetaminophene,

Sulfaguanidine, Verapamil, Terfenadine and Caffeine) were analyzed in the sequence to verify the mass accuracy and the process efficiency during the analytical investigation.

Blank samples were injected at regular intervals, to identify specific ions from the mobile phase, and any contaminants.

The sequences for the analysis were prebuilt in Excel to randomize the samples injections and prevent any spurious classification deriving from the position of the sample in the sequence.

Data analysis

Data pre-processing and pre-treatment

Data were pre-processed using Progenesis QI software (Waters Corporation, Milford, U.S.A.). The so-called Rt_mass variables (where Rt is the retention time and mass is the mass to charge ratio) were generated and two data sets, one for the positive ionization mode (POS data set) and the other for the negative ionization one (NEG data set), were obtained.

Features with more than 20% of missing data and a coefficient of variation greater than 0.20 in the QCs were eliminated. For each variable passing such a filter, missing data were imputed with a random number between zero and the minimum value measured for the variable. To semi-quantify the detected metabolomics features we developed a procedure that uses quality controls with different dilution factors. Data were calibrated on the basis of the local linear regression models obtained considering the trend of the QCs with the run order as previously described.[1] As a consequence, metabolomics variables were expressed using a relative scale. Probabilistic quotient normalization was applied to take into account dilution effects. Data were log-transformed and mean-centered prior to performing data analysis.

Statistical data analysis

Baseline clinical characteristics of the recruited children were analysed by t-test or Mann-Whitney test for continuous normally or non-normally distributed data, respectively, and by Fisher's exact test for categorical variables. Normality of the data was assessed by Shapiro-Wilk test ($p > 0.10$).

In case of differences in the distribution of one or more variables between the two groups a sub-sampling of the children was performed to match responders and non-responders and avoid possible confounding with the outcome (in other word to prevent from interpreting metabolic differences related to these variables as they were related to the outcome, i.e. the response to omalizumab).

Matching procedure

Since the two groups under investigation had a different number of subjects and their populations resulted to be heterogeneous, a matching procedure was applied to avoid bias in the data analysis. The procedure is described in the following.

Given the subject i and the subject j belonging to different groups and P matching variables, the distance between them was defined as $d(i, j) = \sum_{k=1}^P d_k(i, j)$ where, in the case of categorical variables, the contribute was $d_k(i, j) = 0$ if the levels of the descriptor k were the same for the two subjects or $d_k(i, j) = 1$ if the levels were different and, in the case of continuous variables, the contribute was $d_k(i, j) = |x_{ki} - x_{kj}|/s$ if $|x_{ki} - x_{kj}| < s$ and $d_k(i, j) = 1$ if $|x_{ki} - x_{kj}| \geq s$ being x_{ki} the value of the descriptor k for subject i and $s = [\max(x_k) - \min(x_k)]/n$. The parameter n was set equal to 4. As a first step, the

distance matrix between cases and controls was calculated. For each subject of the responder group, the subject of the non-responder group with the minimum distance was selected and the pairs responder/non-responder were sorted on the basis of the increasing distance. The pairs with the greatest distance were iteratively excluded until the set of the selected responders and the set of the selected non-responders did not show significant differences in the descriptor variables.

Approaches used for metabolomic analysis

As for metabolomic data analysis, two different approaches were applied. The first approach (two-group comparison) considers responders and non-responders as two independent groups assuming that each group is homogeneous from a metabolomic standpoint. This approach was based only on univariate methods, because the low numerosity of the non-responder group prevented us from applying multivariate methods. Specifically, Mood's median test controlling the false discovery rate (FDR) by Storey method was applied to compare the medians of responder and non-responder groups for each variable

The second approach (one-class modelling) considers the responders as the reference class, with respect to which non-responders are outliers. This approach was based on univariate and multivariate methods for outlier detection. Principal Component Analysis (PCA) was used to perform multivariate one-class modelling while univariate one-class modelling was performed as follows. For each measured metabolic variable, the data distribution of the responder group was modelled by kernel density estimation using a gaussian kernel. Then, considering the responder reference distribution, for each non-responder we estimated the probability that the measured value of that metabolic variable was an outlier. Controlling FDR by Storey method, the variables that significantly characterised each non-responder as an outlier were highlighted. The threshold to use for false discovery rate depends on the accepted risk of finding false discoveries. Setting the adjusted p-value at 0.05 is sometimes a too conservative approach that may exclude interesting findings. For this reason, we set the cut-off at 0.15. In untargeted metabolomics studies, it is a common practice to use adjusted $p < 0.15$, < 0.10 or < 0.05 . [1,2,3]

Data analysis was performed using in-house R-functions implemented by R 4.0.4 platform (R Foundation for Statistical Computing).

Variable annotation

The relevant variables selected by data analysis were annotated by searching our in-house database of commercial standards, the METLIN metabolite database and the Human Metabolome Database using a different level of confidence.[4] Specifically, level 1 was assigned to compounds with a difference of less than 10 ppm for m/z and 0.2 min for retention time with respect to the standards that were submitted to identical analytical conditions of the current analysis; level 2 was assigned to metabolites with $m/z \leq 10$ ppm with respect to the online databases and retention time similar to that of compounds of the same family present in our in-house database; level 3 to metabolites with $m/z \leq 10$ ppm with respect to the online databases.

RESULTS

57 children with severe allergic asthma were consecutively enrolled in the study.

At recruitment, children had been treated for at least four months with a daily ICS dose ≥ 500 mcg of fluticasone propionate or equivalent.

47 patients were classified as responders to omalizumab and 10 as non-responders. In 5 out of 10 non-responders, omalizumab was stopped after 4 to 9 months of therapy.

A preliminary analysis of the clinical variables at recruitment highlighted a possible selection bias due to a slightly more frequent use of montelukast in the responder group ($p=0.068$). The matching procedure was applied to avoid such bias, assuming a significance level $\alpha=0.15$. As a result, to match responders and non-responders, 5 responders were not considered in the final metabolomic data analysis, and 42 responders and 10 non-responders were compared.

After data pre-processing, two data sets, one composed of 357 Rt_{mass} variables (NEG data set) and the other of 3117 Rt_{mass} variables (POS data set), were obtained. The two data sets were merged and the resulting data set composed of 3474 Rt_{mass} variables was investigated.

Multivariate one-class modelling.

Assuming the responders as the reference class and testing if non-responders belong to that class, one-class modelling based on PCA (4 principal components, $R^2=0.30$, $Q^2=0.10$) showed that 4 non-responders had a different metabolic urinary profile with respect to the responders (figure S1).

The one-class modelling approach highlighted 100 features differently expressed in at least three non-responders with respect to responders. Fifty-five of these features could be annotated (table S1).

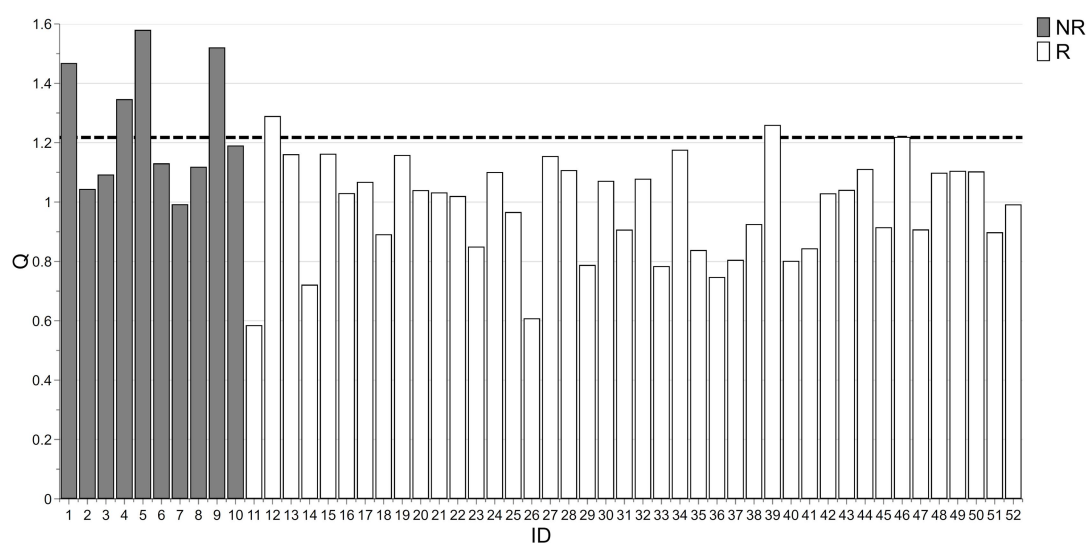


Figure S1. Q plot obtained projecting the observations onto the PCA model of the responder group; Q-distance, reported as bar chart, shows that non-responders have a distribution of Q with higher values than the responders, and 4 non-responders show Q distance greater than the critical value calculated for the responder group assuming a confidence level of 95%. Grey is used for non-responders and white for responders; the dashed line indicates the critical value of Q at the level of 95%.

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Table S1. Annotated variables selected by one-class modelling; ID is the variable identifier coded as [ionization mode][identifier], m/z is the mass to charge ratio, RT is the retention time and level is the annotation level.

ID	m/z	RT	level	annotation	ID	m/z	RT	level	annotation
POS2231	167.0570	2.034	1	Methylxanthine	POS1914	234.0858	1.377	3	Glutamyl-Serine
POS4443	202.0479	3.583	1	Hippuric acid	POS8016	430.2951	5.731	3	Glycocholic Acid
POS408	147.1132	0.517	1	L-Lysine	POS3823	171.1133	3.146	3	Glycyl-L-leucine
NEG172	215.1142	0.746	1	N-Acetyl-Arginine	POS1832	321.0825	1.239	3	Gly-His-OH
POS1992	183.0515	1.500	2	3-Methyluric acid	POS2845	517.4906	2.547	3	Heptadecyl-palmitate
POS5166	319.1658	4.096	3	17 α -Ethinylestradiol	POS7525	151.0757	5.363	3	hydrocinnamic acid
POS3171	282.1175	2.761	3	1-Methyladenosine	POS3143	263.0863	2.729	3	L-beta-aspartyl-L-glutamic acid
POS4460	413.0424	3.583	3	1-Phosphatidyl-D-myo-inositol	POS5317	303.0953	4.172	3	L-beta-aspartyl-L-phenylalanine
POS8538	521.2246	6.128	3	vitamine D3 derivative	POS1771	227.1756	1.174	3	Leucyl-leucine
POS7509	193.1584	5.346	3	2-keto tridecanoic acid	POS5525	243.1340	4.288	3	L-gamma-glutamyl-L-isoleucine
POS3186	129.0554	2.777	3	2-Oxo-4E-hexenoic acid	POS8646	315.1828	6.244	3	Lysyl-Tryptophan
POS2322	197.0672	2.162	3	3,7-Dimethyluric acid	POS3577	224.1285	2.997	3	N-(3-oxo-octanoyl)-homoserine lactone
POS1220	85.0289	0.751	3	3-Butynoate	POS8955	454.2933	6.529	3	PC(13:0/0:0)
POS6878	385.1471	4.988	3	propanoic acid derivative	POS2789	521.2461	2.484	3	PG(8:0/8:0)
POS3554	132.0450	2.986	3	3-Hydroxyphenylglycine	POS4648	231.1131	3.686	3	Phenylalanyl-Threonine
POS8479	225.1848	6.101	3	5,8-Tetradecadienoic acid	POS8490	120.0812	6.106	3	Phenylethanolamine
POS4479	77.0392	3.589	3	5-hexynoic acid	POS2566	199.1081	2.338	3	Prolyl-Threonine
POS1962	243.0735	1.462	3	5-Phosphonooxy-L-lysine	POS2023	130.0506	1.575	3	Pyroglutamic acid
POS7776	359.2618	5.534	3	8-sio Prostaglandin E2 isopropylester	POS5394	174.0556	4.215	3	Quinaldic acid
POS2144	139.0030	1.874	3	Aconitic acid	POS7984	313.1646	5.704	3	Tetranor-PGFM
POS8493	197.0810	6.106	3	α -Methyl-3,4-dihydroxyphenylpropionic acid	POS7999	295.1532	5.715	3	Tetranor-PGFM
POS3181	303.0958	2.766	3	Aspartyl-Phenylalanine	POS1663	143.0345	1.046	3	trans-trans-muconic acid
POS6523	225.1590	4.812	3	Aspergillic acid					
POS4439	105.0340	3.583	3	Benzoic acid					
POS1116	258.1439	0.677	3	E-(gamma-Glutamyl)-lysine					
POS7699	137.1327	5.460	3	Capric acid					
POS5308	170.0453	4.172	3	Dihydrodipicolinic acid					
POS5169	395.0954	4.096	3	Dihydroferulic acid 4-O-glucuronide					
POS2056	149.0238	1.644	3	DL-3,4-Dihydroxymandelic acid					
POS1943	229.0324	1.426	3	DL- α -Lipoic Acid					
POS1738	234.0436	1.131	3	Dopamine 4-sulfate					
POS7565	464.1874	5.390	3	Etodolacglucuronide					
POS5048	259.1079	3.995	3	Glutamylphenylalanine					