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Serum metabolic signature of binge-like palatable food consumption in female rats by nuclear magnetic resonance spectroscopy

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# **Serum Metabolic Signature of Binge-Like Palatable Food Consumption in Female Rats by Nuclear Magnetic Resonance Spectroscopy**

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## **Abstract.**

Maladaptive eating behavior is a growing public health problem and compulsively eating excessive food in a short time, or binge eating, is a key symptom of many eating disorders. In order to investigate the binge-like eating behavior in female rats, induced by intermittent food restrictions/refeeding and frustration stress, we analyzed for the first time the metabolic profile obtained from serum of rats, through the Nuclear Magnetic Resonance (NMR) spectroscopy. In this experimental protocol, rats were exposed to chow food restricting/refeeding and frustration stress manipulation. This stress procedure consists of 15 min exposure to the odor and sight of a familiar chocolate paste, without access to it, just before offering the palatable food. In this model, a “binge-eating episode” was considered the significantly higher palatable food consumption within 2 hours in restricted and stressed rats (R + S) than in the other three experimental groups: rats with no food restriction and no stress (NR + NS), only stressed rats (NR + S) or only restricted (R + NS). Serum samples from these four different rats groups were collected. The statistical analysis of the <sup>1</sup>H NMR spectral profiles of the four sets of samples pointed to O- and N-acetyl glycoproteins as the main biomarker for the discrimination of restriction effects. Other metabolites, such as Thr, Gly, Gln, Ac, Pyr and Lac, showed trends that may be useful to understand metabolic pathways involved in eating disorders. This study suggested that NMR-based metabolomics is a suitable approach to detect biomarkers related to binge eating behavior.

**Keywords.** Binge-eating behavior, metabolomics, NMR, animal model, stress, palatable food, female rats, food restriction

## **Introduction**

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The urge to compulsively overeat certain food in a short time, leading to an episode of binge eating, is a key symptom in major types of eating disorders<sup>1</sup>, including bulimia nervosa (BN), binge/purging subtype of anorexia nervosa (AN) and binge-eating disorder (BED). Eating disorders are highly prevalent in adolescents, especially in young women, and rates are increasing worldwide.<sup>2,3</sup> Moreover they are associated with serious psychiatric complications, such as depression or anxiety, that impact on quality of life<sup>4</sup> and increase mortality.<sup>5</sup> BED represents the most prevalent eating disorder<sup>6,7</sup> and is characterized by loss of control during which people eat large amounts of highly palatable food without engaging in regular compensatory behaviours after bingeing, such as induced vomiting, laxative misuse or excessive exercise for controlling weight gain.<sup>1</sup>

Data suggest that individuals with BED have greater cognitive attentional biases towards food, decreased reward sensitivities, and altered brain activation in regions associated with impulsivity and compulsivity, compared to individuals without BED.<sup>7</sup> The same brain reward deficit is detected with drug abuse and is considered to be an important trigger in the progression from normal to compulsive intake of drugs or food, supporting the “food addiction” hypothesis in humans<sup>8,9</sup> and animals.<sup>10-12</sup>

Despite this evidence, therapies are extremely limited and the only medication approved by the Food and Drug Administration (FDA) is Lisdexamfetamine dimesylate (LDX, Vyvanse®).<sup>13</sup> Adverse reactions associated with LDX include insomnia, dry mouth, diarrhea, anxiety, agitation, increased blood pressure; thus patients with hypertension, coronary artery disease, diabetes, psychiatric disorders or substance abuse are not eligible for this treatment.<sup>13,14</sup>

In this context, animal models are essential to facilitate the study of underlying behavioral and physiological mechanisms in binge-eating behavior. Different binge-eating animal models are available, in which several environmental conditions (acute or chronic stress, food restriction, and limited-access to palatable food) are recruited to elicit aberrant feeding behavior on palatable food, mimicking clinical features of eating disorders.<sup>15-18</sup> The aim of this study was to evaluate the serum metabolic profile, using a well-characterized animal model of binge eating behavior,<sup>19-24</sup> hypothesizing that biochemical changes are associated with different eating behaviors in the animals. Female rats were chosen because binge eating disorder and bulimia nervosa are more prevalent in women than in men.<sup>6</sup>

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In this experimental protocol, rats were exposed to chow food restricting/refeeding and frustration stress manipulation.<sup>25</sup> This stress procedure, which has already shown to increase corticosterone levels in rats,<sup>26-28</sup> consisted of 15 min exposure to the odor and sight of a familiar chocolate paste without access to it, just before offering the palatable food. In this model, “binge-eating episode” was considered the significantly higher palatable food consumption within 2 h observed in restricted and stressed rats but not in the other three experimental groups: rats with no food restriction and no stress, rats with only stress, or rats with only food restriction. Thus, like in humans, the synergistic relationship between stress and caloric restriction affects both the qualitative and the quantitative aspects of eating patterns,<sup>18,29</sup> reprogramming stress and orexigenic pathways.<sup>30-32</sup> A further element of validity in this animal model is the influence of ovarian hormones on binge-like eating behavior<sup>33,34</sup>, as in women.<sup>35,36</sup>

Performing the NMR analysis in these rats could be a useful approach to provide new knowledge on compulsive eating, identifying potential biochemical markers that contribute to this maladaptive eating behavior. There are limited studies on metabolome measurements in eating disorders<sup>37,38</sup> that are especially focused on AN.<sup>37,39-41</sup> Recent studies suggest that metabolomics play an interesting role in distinguishing normal-weight and obese individuals and could be considered an encouraging clinically diagnostic strategy for obesity state.<sup>42-45</sup> Moreover, the analysis of NMR spectra of urine revealed a metabolic separation in mice under high-fat diets or high-carbohydrate diets, inferring that these different diets induced obesity through distinct mechanisms.<sup>46</sup>

The NMR spectra constitute a “fingerprint” of the NMR-detectable part of the whole metabolome. Metabolomics research is a consolidated area aimed at detecting the pool of metabolites in biological systems. The metabolomic profile is composed of low-molecular weight compounds that are involved in various metabolic pathways. These small molecules, including lipids, sugars, amino acids, nucleotides and a series of different organic molecules, are reactants, intermediates, or products of biochemical reactions, and are the building blocks of other molecules such as proteins, nucleic acids and cell membranes. Most metabolomics-profile studies apply NMR on common biological fluids, such as urine and serum/plasma that are obtainable in a non- or minimally invasive way and are easily

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available. Blood serum is a primary carrier of small molecules, transporting all the molecules that are secreted, excreted or discarded by the body.

Using NMR spectroscopy in this study, we investigated, the metabolic profile obtained from serum of female rats subject to the binge eating model.

## **EXPERIMENTAL PROCEDURES**

### **Animal Model and Sample collection**

Thirty-two female Sprague-Dawley rats (Charles River, Italy), fifty-two days old were submitted to the binge eating protocol as described in previous works<sup>25,47-49</sup> and in the Supporting Information.

The rats were divided into four different experimental groups: 1. NR + NS (non-restricted and non-stressed rats, the control group); 2. NR + S (non-restricted and exposed to stress); 3. R + NS (restricted and non-stressed); 4. R + S (restricted and stressed).

Briefly, female rats were exposed (or not exposed) for 24 days to three eight-day cycles of food restriction/refeeding (66% of chow intake on days 1–4 and free feeding on days 5–8 of each cycle) during which they were given access to palatable food for 2 h during the light cycle (light on at 9 a.m.) on days 5–6 and 13–14 of the first two cycles. In the third cycle the rats were exposed (or not exposed) to 66% of chow intake on days 17–20 and free feeding on days 21–24. On the binge intake test day (day 25) rats were sacrificed immediately after receiving frustration stress. Vaginal smears and blood samples were collected. Vaginal secretions were collected with a cotton swab moistened with physiological saline (NaCl 0.9%) by superficially inserting the tip into the rat vagina, then placed on a glass slide and examined under a microscope using standard criteria.<sup>50</sup> Serum was separated from the blood samples after coagulation. After standing for 15 min at room temperature and then 1 h on ice, samples were centrifuged at 4° C for 15 min at 1000 x g and serum (supernatant) was collected and kept at -80°C until NMR analysis. Only the serum samples of non-estrus rats were used for NMR analysis, since we previously demonstrated that binge eating episodes did not occur during the estrous phase in this animal model.<sup>34</sup>

### **NMR analysis**

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The experiments were performed using a Bruker Avance400 spectrometer with a 5mm BBI probe, working at 400.13 MHz on  $^1\text{H}$ .

Frozen samples were thawed at room temperature and 100  $\mu\text{l}$  of  $\text{D}_2\text{O}$  were added to 400  $\mu\text{l}$  of serum. All  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were acquired at 298 K. The serum spectra were referenced to the alanine doublet at 1.48 ppm. For each sample three  $^1\text{H}$  NMR spectra were acquired using:

- i) a composite pulse sequence (zgcprr), with 3.0 s water-presaturation during relaxation delay, 13.02 kHz spectral width, 32k data points, 32 scans, 4 dummy scans; the free induction decay (FID) was zero-filled once and Fourier transformed with a line broadening of 0.5 Hz;
- ii) a water-suppressed spin-echo Carr-Purcell-Meiboom-Gill (CPMG) sequence (cpmgpr), with 3.0 s water presaturation during relaxation delay, 1 ms echo time ( $\tau$ ), and 90 ms total spin-spin relaxation delay ( $2n\tau$ ), 13.02 kHz spectral width, 32k data points, 64 scans, 4 dummy scans; FID was zero-filled once and Fourier transformed with a line broadening of 0.5;
- iii) a sequence for diffusion measurements based on stimulated echo and bipolar-gradient pulses (ledbpgp2s1d, hereinafter simple referred to as led in the text), with big delta 50 ms, eddy current delay  $T_e$  5 ms, little delta  $2 \times 2$  ms, sine-shaped gradient with 32 G/cm followed by a 200  $\mu\text{s}$  delay for gradient recovery, 12.0 kHz spectral width, 8k data points, 64 scans, 4 dummy scans.

Two-dimensional (2D) experiments COSY, TOCSY and HSQC were performed to characterize the complete metabolome of serum samples<sup>51</sup> (for details see the supporting information).

## Data processing

The NMR spectra were pre-processed using the software package TOPSPIN (Version 3.2, Bruker Biospin, Germany). All zgcprr and cpmg  $^1\text{H}$  NMR spectra were subjected to 0.5 Hz exponential line broadening (for NMR led spectra the line broadening used was 5 Hz), zero-filled to 64k points prior to Fourier transformation and then phased and baseline corrected

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manually. After these preliminary processing, all spectra (from zgcprr, cpmg and led) were prepared for chemometric analysis using MNOVA software package (MestReNova, ver. 11.0, 4-18998, 2017 Mestrelab Research S. L., Santiago de Compostela, Spain). The  $\delta$  5.5–0.5 ppm spectral region was aligned after splitting it in 13 sub-regions, normalized with respect to the total area (100) and finally a small binning process (0.002 ppm, 1.6 Hz) was performed. The spectral region around the water pre-saturated signal ( $\delta$  4.68–5.20 ppm) was discarded. For the quantitative analysis, 9 spectral regions of cpmg and led spectra were selected and subjected to spectral deconvolution.

### Statistical analysis

The normalized data set was used to perform multivariate statistical analysis. First, principal components analysis (PCA), an unsupervised multivariate ~~pattern recognition~~ method, was performed to examine the intrinsic variation in the data set. Next, a supervised projection to partial least squares-discriminant analysis (PLS-DA) was performed for class discrimination. A 7-fold cross validation and 1000 permutation test were used to assess the quality of PLS-DA model.

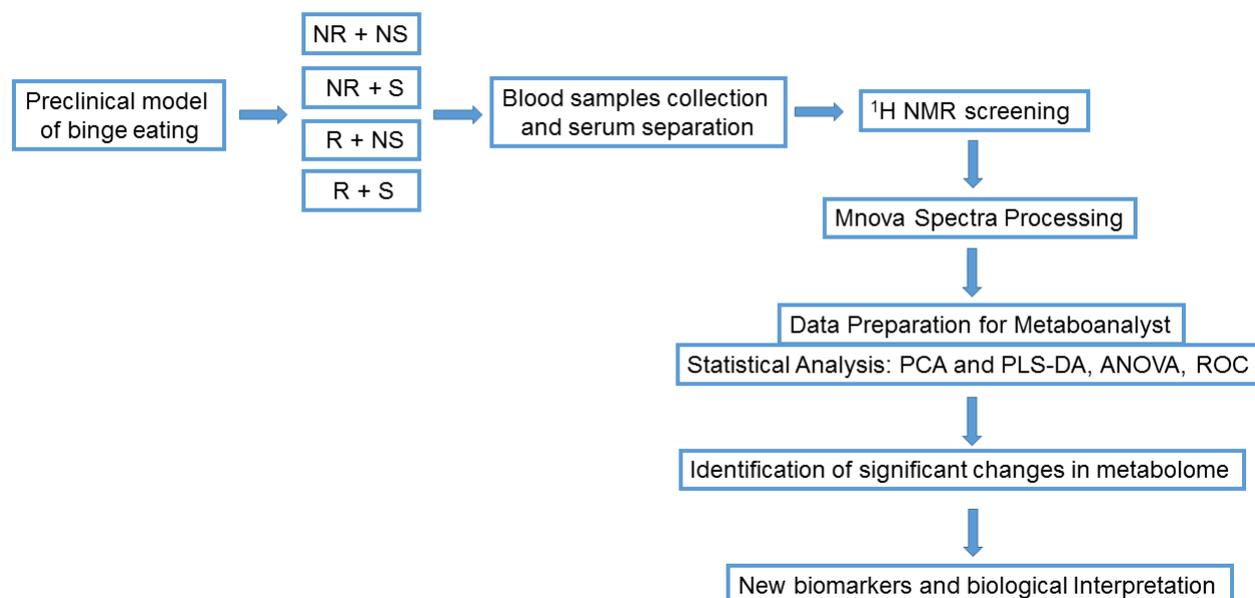
The metabolite signals highlighted by multivariate analysis were deconvoluted and a one-way ANOVA study was performed in all four classes to test statistically significant differences in metabolite contents. Moreover, the potential biomarkers underwent receiver operating characteristic (ROC) analysis. By analyzing the area under the receiver-operating curve (AUC), we identified metabolites that could classify among the 4 groups. We focused on metabolites with an  $AUC > 0.7$  as potential biomarkers. All of these analyses were performed using MetaboAnalyst 4.0, a web-based metabolomics data analysis software.<sup>52</sup>

The flowchart details the experimental design of our study are reported in Scheme 1.

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## METABOLOMICS FLOWCHART



### Scheme 1

## RESULTS

### *NMR Metabolites Characterization*

Twenty-four rat serum samples were used in the NMR analysis. A total of eight rats were excluded from the analysis because they were in estrous phase of the ovarian cycle. The four groups of serum samples were characterized and several metabolites were detected. In Figure 1 three different NMR spectra of representative sample are reported. Figure 1A is a water-presaturated <sup>1</sup>H NMR spectrum and shows the complete serum metabolome. It is a complex spectrum dominated by lipids and substantial background signal due to macromolecules. An experiment more suitable for detecting the narrow signals from small metabolites is the <sup>1</sup>H CPMG sequence that reduces lipids and macromolecule resonances (Figure 1B). The small molecules detected in serum are amino acids, sugars, osmolites, and polyols. Figure 1C displays contributions from mobile lipids and macromolecules, derived from a diffusion-edited experiment. The labels in Figure 1 were chosen to indicate the most abundant and visible metabolites in each spectrum. The complete assignment of signals requires the acquisition of selected 2D experiments. COSY, TOCSY, and HSQC spectra

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were acquired and analyzed, and several metabolites were identified and assigned on the basis of H,H and H,C correlations and by comparison with literature data. The results from COSY and HSQC are reported in Figure S1. The list of all the detected compounds is provided in the supporting material Table S1.

<Figure 1>

Figure 1. NMR profiling of serum. 1D  $^1\text{H}$  NMR spectra of serum obtained with (A) water-presaturation pulse sequence with composite pulse; (B) CPMG sequence; (C) diffusion-edited spectrum. Labels: Ala, alanine; Gln, glutamine; Val, valine;  $\alpha$ -Glc,  $\alpha$ -glucose;  $\beta$ -Glc,  $\beta$ -glucose; Lac, lactate; Cr, creatine; FA, fatty acids; MM, macromolecules; PL, phospholipids, *N*, *O*-acetyl are related to glycoprotein.

Among the most interesting signals that are labelled in Figure 1B (CPMG) there are two singlets at 2.05 (s) and 2.14 (s) ppm. These two resonances were assigned on the basis of their specific H and C chemical shifts to acetyl groups bound to *N*- and *O*-, respectively, and they have been identified as mobile carbohydrate side chains of glycoproteins.<sup>53</sup>

## Multivariate analysis

### Chemometric analysis

#### *Small Metabolites*

Chemometric analysis was performed on samples of all four groups of rats. The unsupervised PCA analysis was applied to the  $^1\text{H}$  CPMG spectra (total spin-echo time 90ms) in order to see if some condition-dependent clustering occurred spontaneously and to better highlight metabolite variations. The first three principal components account for 84.4% of the total variance and allow us to deduce information on the metabolites that can be responsible for the observed clustering. The PC2, PC3 scores plot, reported in Figure 2A, shows a good separation between NR + NS (control group) and R + S (binge-eating group). Moreover, the NR + S group is more scattered along PC2, whereas R + NS samples fall at negative PC2 values similar to the R + S ones. The PLS-DA scores plot (Figure 2B) reveals satisfactory

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discrimination among three of the 4 groups, with the NR + S group overlapping the NR + NS group. The validity of the PLS-DA model against over-fitting was assessed by the parameters R<sup>2</sup> (0.75), and the predictive ability was described by Q<sup>2</sup> (0.50), data are reported in Figure S2. Theoretically, the PLS-DA model is better when Q<sup>2</sup> is close to R<sup>2</sup> and R<sup>2</sup> is close to one. A permutation test (n = 1000) was performed to assure the predictive capacity of the PLS-DA model. The observed p value via permutation testing was 0.003, thus confirming the validity of the PLS-DA model. In particular, R + S and R + NS are well separated from NR + NS. NR + S results also in this case scattered on the two components.

< Figure 2 >

Figure 2. (A) PC2, PC3 PCA and (B) LV1, LV2 PLS-DA scores plot obtained on CPMG spectra are reported. Groups: ● (NR + NS), ▲ (NR + S), ▲ (R + NS) and ● (R + S). The ellipses mark the 95% confidence regions for each group.

The loadings plot of PC2 and PC3 (Fig. S3A) and of PLS-DA (LV1 and LV2 Fig. S3B) visualize the influence of each metabolite on the separation between the groups. Positive PC3 values, corresponding to higher levels of small metabolites such as Ala, Val, leucine (Leu), acetate (Ac) and Lac, characterize the R + S group, together with negative PC2 values, contributed mainly by fatty acid (FA) residues. As for PLS-DA positive peaks (in particular cholesterol Chol and FA) in the loadings plot of LV1 (Fig. S3B) discriminate R + NS and R + S groups from NR + NS and NR + S. The negative loadings of LV2 shows that both O- and N- acetyl signals can be important for the discrimination between NR + NS, NR + S and R + S, R + NS classes. This is less evident when PCA loading are considered.

#### *Analysis of Lipids and Macromolecules*

PCA and PLS-DA analyses were also applied to <sup>1</sup>H led (diffusion edited) spectra in order to see if some condition-dependent clustering occurred, considering lipids and

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macromolecules present in the samples. In Figure 3, we observed that NR + NS and NR + S are characterized by more positive values of PC1 and PC4 in PCA scores plot and by more negative values of LV1 and LV2 in PLS-DA scores plot, with respect to R + S and R + NS groups. Diffusion-edited spectra partially discriminate the 4 groups, although the validity of the PLS-DA model as shown by Q2 and R2 values is low (Figure S4). PC1 and LV1 loadings plots are dominated by macromolecule signals, among which the two O- and N-acetyl resonances coming from glycoproteins can be clearly distinguished (Figure S5), whereas PC4 and LV2 loadings plots highlight signals due to unsaturated FA and phospholipids (Figure S5).

< Figure 3 >

Figure 3. Scores plot of PCA (A) and PLS-DA (B) analyses on diffusion edited spectra are reported. Groups: ● (NR + NS), ▲ (NR + S), ▲ (R + NS) and ● (R + S). The ellipses mark the 95% confidence regions for each group.

### Quantitative Analysis

#### *Small Metabolites*

After the multivariate analysis on the spectra, we ran a comparison among groups based on an ANOVA test on the signals of the metabolites obtained by deconvolution. The only statistically significant differences were those of glycoproteins at 2.05 and 2.14 ppm ( $p < 0.05$ ). A post-hoc test based on Tukey's Honest Significant Difference showed that this difference is significant when samples NR + NS and NR + S are compared to R + NS and R + S, but not when NR + NS group is compared to NR + S and when R + NS class is compared to R + S. (Figure S6 and Table 2S).

We used classical univariate ROC analysis to test the diagnostic value of each potential binge eating biomarker (Fig. 4). Besides glycoproteins other metabolites that were involved in the partial separation of the groups in multivariate analysis, such as Lac, Pyr, Gln, Gly, Ac, Myo, and Cr were tested.

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<Figure 4>

Figure 4. ROC analysis between groups NR + NS and R + S. Metabolites with AUC > 0.7 are selected.

Table 1 reports ROC analysis on potential biomarkers, derived from multivariate analysis, performed by comparing two by two for all groups.

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<b>COMPARISON</b>	<b>METABOLITE</b>	<b>AUC</b>	<b>T-TEST</b>	<b>LOG<sub>2</sub>FC</b>
<b>NR + NS vs R + S</b>	Glycoprotein	1.00	0.00008	2.457
	Thr	0.88	0.070	-1.058
	Gln	0.86	0.028	0.240
	Gly	0.74	0.106	0.400
<b>NR + NS vs R + NS</b>	Glycoprotein	1.00	0.0003	2.298
	Thr	1.00	0.001	-1.161
	Pyr	0.86	0.015	1.363
	Ac	0.86	0.058	0.704
	Gly	0.77	0.197	0.550
	Gln	0.77	0.083	0.591
	PC	0.74	0.439	0.424
<b>NR + NS vs NR + S</b>	Thr	0.88	0.017	-0.877
	Ac	0.86	0.046	0.829
	Myo	0.74	0.055	-0.395
<b>R + S vs R + NS</b>	Ac	0.83	0.097	0.624
	Pyr	0.80	0.078	1.133
	Lac	0.80	0.129	0.530
	Thr	0.77	0.830	-0.104
<b>R + S vs NR + S</b>	Glycoprotein	0.97	0.005	-2.230
	Myo	0.80	0.086	-0.537
	Ac	0.80	0.081	0.753
<b>NR + S vs R + NS</b>	Glycoprotein	1.00	0.012	2.271
	Myo	0.77	0.166	0.651
	Thr	0.73	0.142	0.605
	Lac	0.73	0.221	0.479
	Pyr	0.70	0.140	1.029
	Glc	0.70	0.238	1.399
	Cr	0.70	0.608	0.340

Table 1. Comparison between groups: metabolites that present AUC>0.7 are reported with relative t-test value and Log2 fold change (FC). Threonine (Thr); pyruvate (Pyr); acetate (Ac); creatine (Cr); glucose (Glc); glutamine (Gln); glycine (Gly); lactate (Lac); myo-inositol (Myo).

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We also performed multivariate ROC analysis on all groups of samples compared two by two. Figure S7 shows the predictive accuracy of biomarker models with an increasing number of features. Figure S8 reports PCA, PLS-DA, results for analysis of small molecules between NR + NS and R + S groups.

#### *Analysis of Lipids and Macromolecules*

The signals that characterize the discrimination between NR + NS and R + S are derived from FA (Figure 5). FA content is higher in the NR + NS group, and decreases in rats after the treatment of restriction and stress R + S. As was done for the small molecules, the univariate ROC analysis was performed on all groups taken two by two and the major significant data are reported in Table 3S. Multivariate ROC analysis was applied on all groups compared two by two, and Figure S9 shows the predictive accuracy of biomarker models with an increasing number of features for lipids and macromolecules. Figure S10 reports PCA, PLS-DA, results for the analysis of lipids and macromolecules comparing NR + NS and R + S groups.

<Figure 5>

Figure 5. ROC analysis between groups NR + NS and R + S. Fatty acids signals with AUC > 0.8 are selected.

#### **Discussion**

NMR represents a promising research tool to identify and measure a large number of metabolites in biological samples, which could be useful for characterizing the metabolic changes that occur in binge eating behavior.

In this work, we explored the metabolic fingerprint and biomarkers using NMR in a well-characterized animal model of binge eating behavior.

Targeting serum metabolomics in female rats under a binge-eating protocol, we performed qualitative and quantitative analyses of small metabolites, lipids, and macromolecules with interesting results. We studied only female rats in accordance with the high prevalence of eating disorders among young adolescents and young adult women. This could represent a possible limitation of the present work.<sup>3</sup>

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This NMR metabolomic survey allowed for the identification of small-molecule metabolites such as glycoproteins, Thr, Gly, Gln, Lac; the homeostasis of which became altered during the development of binge-like eating behavior.

Although most of the broad protein resonances were suppressed by the CPMG pulse sequence in the small metabolite analysis, a significant presence of two signals was detected: N-acetyl and O-Acetyl glycoproteins at 2.05 and 2.14 ppm respectively. We observed that these glycoproteins are important for the discrimination among the four groups (Table 1). In particular, the level of glycoproteins was very low when comparing the binge eating (R + S) versus control (NR + NS) groups. The difference is also significant when R + NS and R + S samples were compared to NR + NS and NR + S, but not when the NR + NS group was compared to NR + S, or when R + NS was compared to R + S. This significant variation prompted us to analyze the potential meaning and role of these compounds, which we hypothesize is likely related to the effect of restriction. Acetyl-glycoproteins are acute-phase proteins that act as inflammatory mediators.<sup>54</sup>

The level of these proteins increase immediately in response to external or internal challenges, such as infection, inflammation, and stress.<sup>53</sup> Previous studies have shown that a disturbance of carbohydrate metabolism may be related to an abnormal O-acetyl glycoprotein response.<sup>55,56</sup> The change in the level of O-acetyl glycoprotein is related to disorder in energy metabolism. These N-glycans and O-glycans combine with different protein amino acids. Brockhausen and co-workers demonstrated that the biosynthesis of glycoproteins (N- and O-glycan) is controlled via different mechanisms including at the level of gene expression, mRNA, enzyme protein activity and localization, including substrate and cofactor concentration at the site of synthesis. The glycosylation can be disease specific and may be associated with the state of the disease.<sup>57</sup>

We also found that the level of Gly decreases comparing the NR + NS control group to the R + S group. Gly is an important building block for proteins and is also necessary for multiple metabolic pathways, such as glutathione (GSH) synthesis and regulation of one-carbon metabolism. In a recent review, the role of Gly has been summarized and its low plasma concentration has been associated with obesity and related metabolic disorders.<sup>44</sup> Thus chronic reduction in Gly in binge-eating rats (R + S) could increase the risk to develop obesity, taking into account that BED is often associated with overweight<sup>58,59</sup> and co-

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occurring pathologies such as diabetes or metabolic syndrome.<sup>60</sup> Additionally, the impact of Gly on adiposity and insulin resistance can potentially be explained by improved insulin sensitivity as reported,<sup>62</sup> and/or increased antioxidative and anti-inflammatory capacity.<sup>63</sup>

Thr is a polar amino acid that has high water solubility, and in our study discriminates the control group from to the test groups. Thr is low in the NR + NS and increases in R + S group as well as the NR + S and R + NS groups. We hypothesize that the combination of food restriction and stress may activate the Thr pathway as a defence mechanism against obesity. A recent study has reported the role and effect of Thr in eating disorders,<sup>64</sup> and has demonstrated the anti-obesity effect of a Thr derivative. Another study reported the effect of Thr in the reduction of fat accumulation,<sup>65</sup> and this in accordance with the low level of FA that we observed in the R + S group.

FA signals, including PC and GPC, decrease in R +S. FA are one of the main energy sources of the body, and they can release a large quantity of energy when there is sufficient oxygen supply. Alterations of these metabolites can cause the development of energy barriers in anorexia nervosa patients. Some studies have revealed that the levels of glycerol and FA increase significantly in obese rats and depend on an estrogen deficiency.<sup>66</sup> On the contrary, our data are consistent with previous reports showing that AA provision inhibits fat deposition by regulating FA biogenesis and the hydrolysis of triacylglycerol,<sup>67,68</sup> and increasing FA oxidation in white adipose tissues resulting in only a small proportion of fatty acid release.<sup>69</sup>

Minor changes were observed on Lac, Pyr, and Glc levels in our groups. These metabolites are involved in Glc metabolism, and Lac has been implicated in the pathogenesis of insulin resistance and obesity.<sup>70</sup> In particular we observed a Lac increase in the R + S group. On the basis of a previous study we can speculate that blood Lac may accumulate in rats with binge eating.<sup>71</sup>

## **Conclusion**

Using a metabolomics approach, we characterized the metabolic profile of a rat model of binge-eating behavior. Serum NMR spectroscopy revealed changes in the levels of

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metabolites that may represent early metabolic perturbations in R + S rats, possibly leading to overconsumption of food and maladaptive feeding behavior. With this approach we propose that metabolites, such as glycoproteins, also in addition to threonine, glycine, glutamine, acetate and lactate, may represent important biomarkers for binge-eating disorder. These results may help to attain a better understanding of eating disorders and to unmask the metabolic pathways related to them. Future similar studies using a larger number of samples will be useful to validate the biomarkers involved in the mechanism of binge eating and potentially used as therapeutic targets.

### **Abbreviations**

Ac acetate

Ala alanine

AN anorexia nervosa

AUC area under the curve

$\alpha$ -Glc  $\alpha$ -glucose

$\beta$ -Glc  $\beta$ -glucose

BN bulimia nervosa

BED binge-eating disorder

Chol cholesterol ester

COSY COrrrelation SpectroscopY

CPMGPR Carr-Purcell-Meiboom-Gill with presaturation

Cr creatine

$\delta$  NMR chemical shift in ppm

FA fatty acid

FDA food and drug administration

Gln glutamine

Glu glutamate

Gly glycine

GPC glycerophosphocholine

GSH glutathione

HR-MAS high-resonance magic-angle spinning

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HSQC heteronuclear single quantum coherence  
Lac lactate  
LDX lisdexamfetamine dimesylate  
Leu leucine  
Lip lipid  
MM macromolecules  
Myo myo-inositol  
N-Ac N-acetyl  
NS + NR non-restricted and non-stressed rats  
NR + S rats non restricted but only exposed to stress  
R + NS restricted and not stressed rats  
R + S restricted and stressed rats  
PC phosphocholine  
PCA principal component analysis  
PL phospholipids  
PLS-DA partial-least square discriminant analysis  
ROC receiver operating characteristic  
SNR signal-to-noise ratio  
TOCSY TOtal Correlation SpectroscopY  
Val valine

Supplementary Materials include Metabolite table, and Figures with representative  $^1\text{H}$  and  $^{13}\text{C}$  HR-MAS NMR spectra and more statistical data analysis.

### **Author Contributions**

VR, SA and CC designed and constructed the study, MVMDB, LB, and CC performed the *in vivo* study, SA, and CB prepared blood samples. VR carried out the NMR measurements, statistical analysis and analysed data. VR writing - original draft and figures. SA edited the figures. VR, SA, AM, MVMDB, CB, and CC co-wrote, edited and revised manuscript. All authors have given approval to the final version of the manuscript. CC, AM, and NB provided resources.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## DATA AND CODE AVAILABILITY STATEMENT

The source code and the datasets generated during the current study are available from the corresponding author on reasonable request.

**Data and code availability statement:** The source code and the datasets generated during the current study are available from the corresponding author on reasonable request.

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## Figures

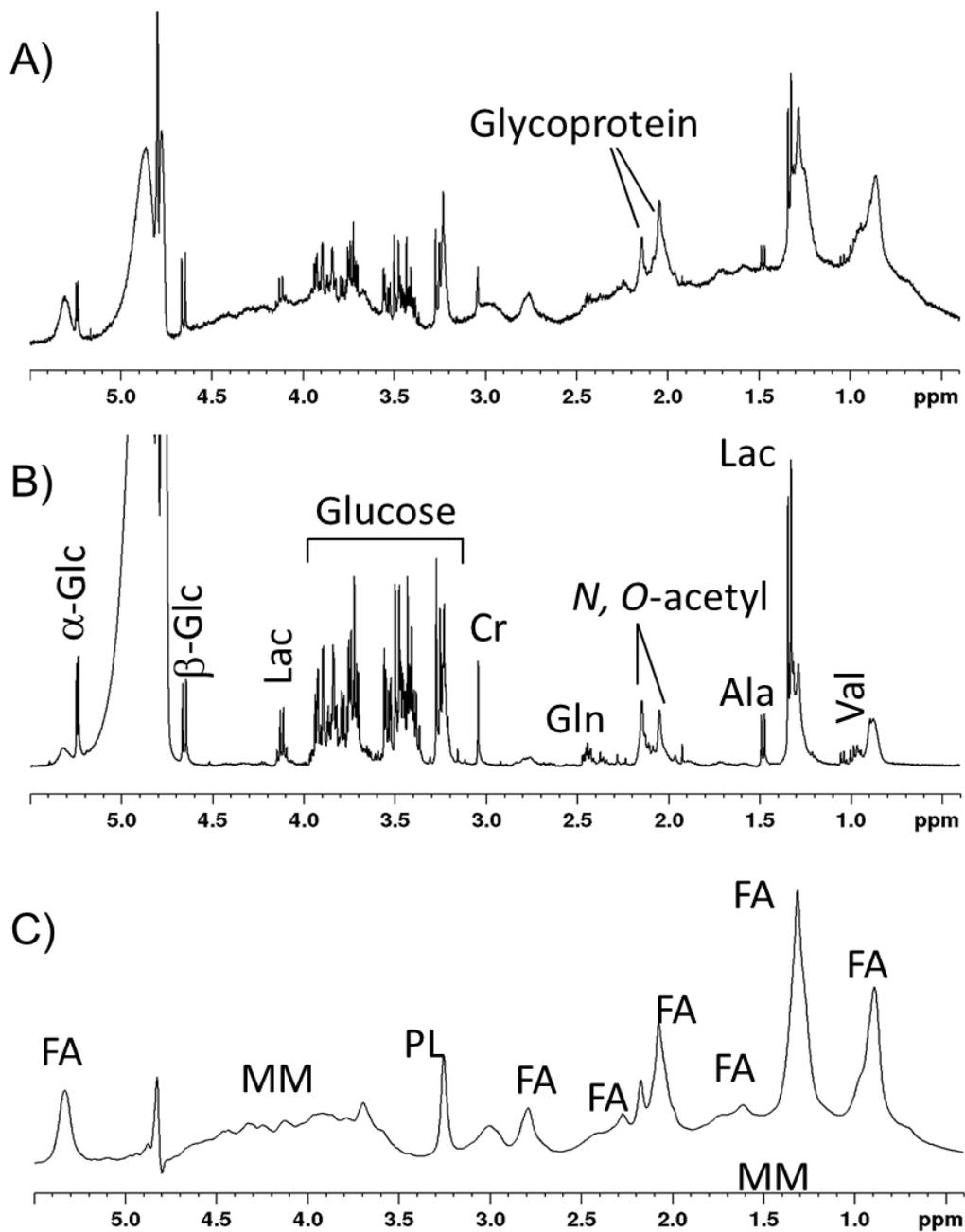


Figure 1. NMR profiling of serum. 1D  $^1\text{H}$  NMR spectra of serum obtained with (A) water-presaturation pulse sequence with composite pulse; (B) CPMG sequence; (C) diffusion-edited spectrum. Labels: Ala, alanine; Gln, glutamine; Val, valine;  $\alpha$ -Glc,  $\alpha$ -glucose;  $\beta$ -Glc,  $\beta$ -glucose; Lac, lactate; Cr,

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creatine; FA, fatty acids; MM, macromolecules; PL, phospholipids, *N*, *O*-acetyl are related to glycoprotein.

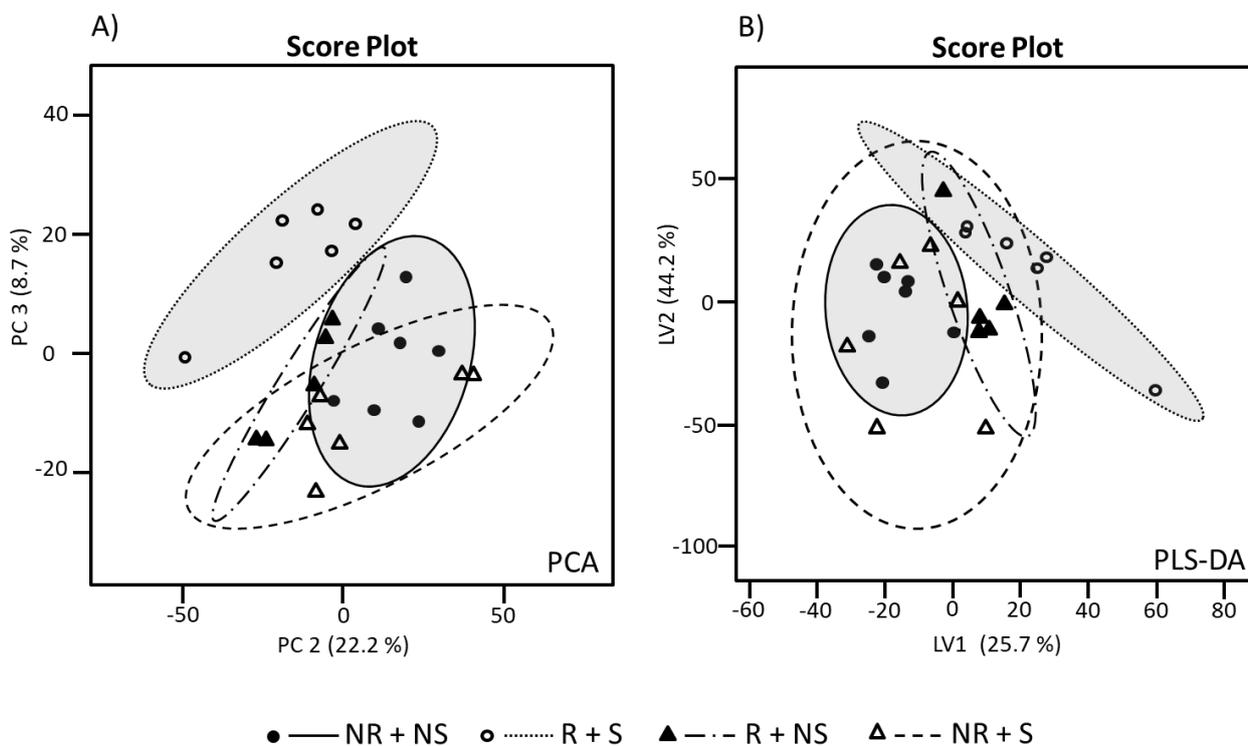


Figure 2. (A) PC2, PC3 PCA and (B) LV1, LV2 PLS-DA scores plot obtained on CPMG spectra are reported. Groups: ● (NR + NS), ▲ (NR + S), ▲ (R + NS) and ○ (R + S). The ellipses mark the 95% confidence regions for each group.

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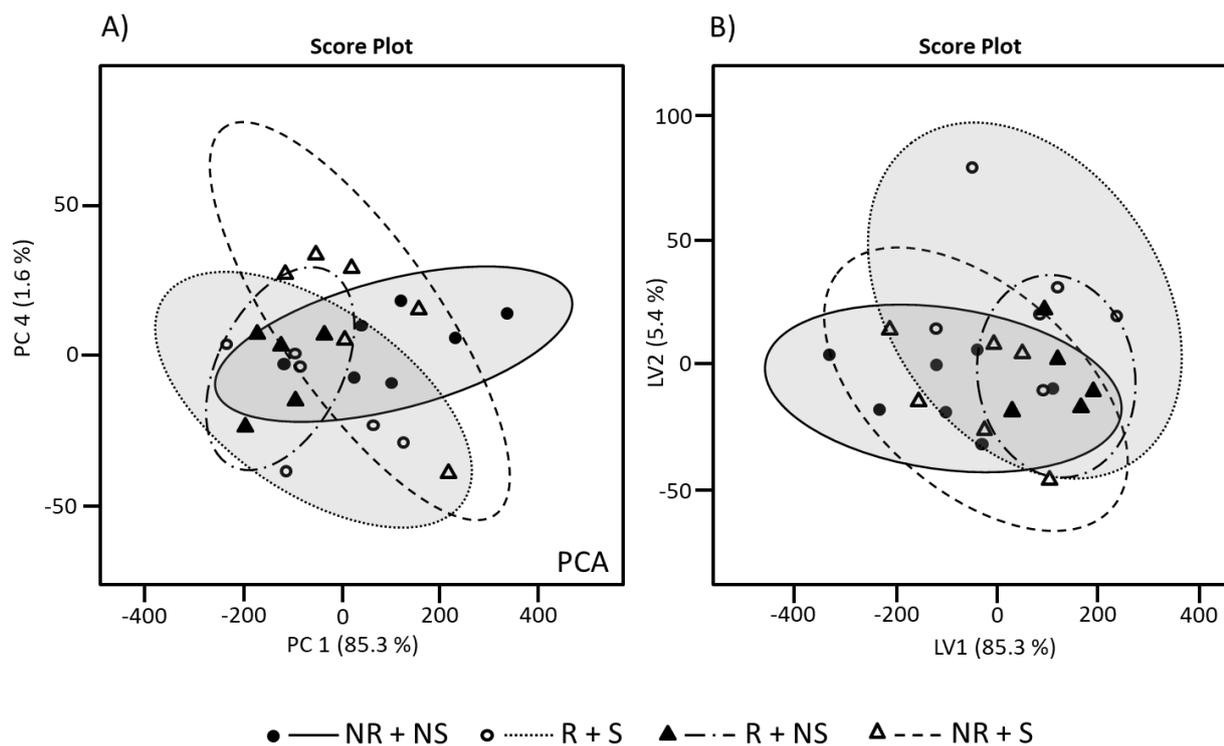


Figure 3. Scores plot of PCA (A) and PLS-DA (B) analysis on diffusion edited spectra are reported. Groups: ● (NR + NS), ▲ (NR + S), ▲ (R + NS) and ○ (R + S). The ellipses mark the 95% confidence regions for each group.

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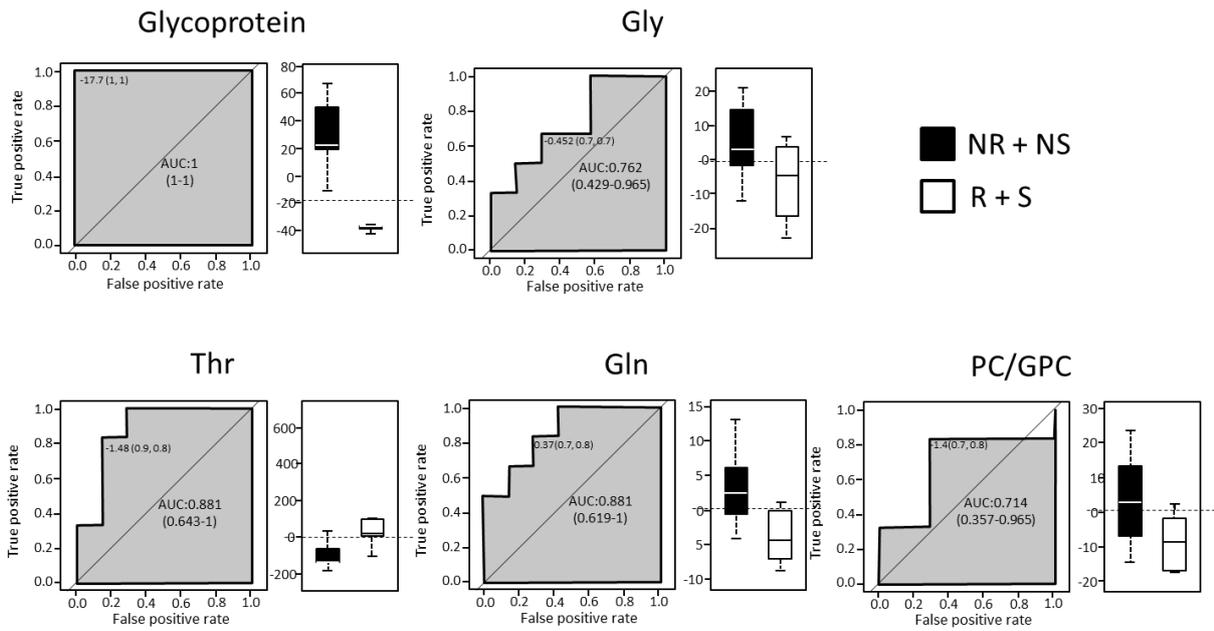


Figure 4. ROC analysis between groups NR + NS and R + S. Metabolites with AUC > 0.7 are selected.

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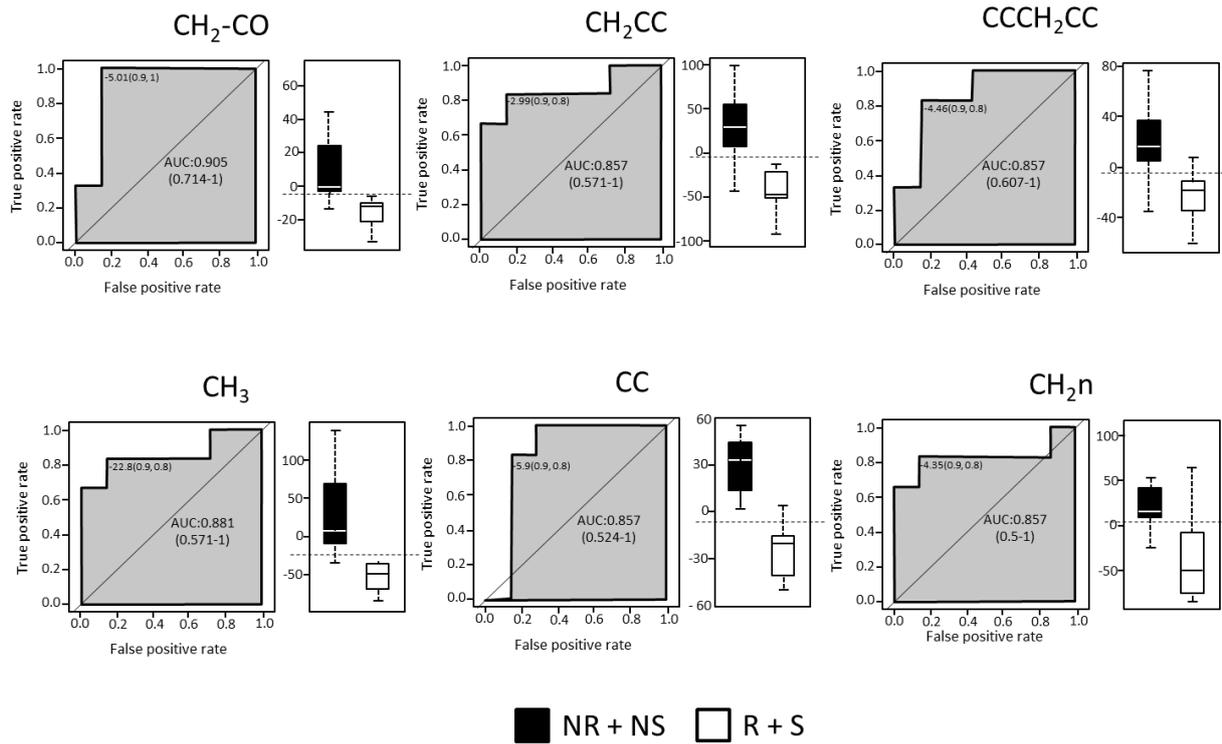


Figure 5. ROC analysis between groups NR + NS and R + S. Fatty acids signals with AUC > 0.8 are selected.

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