Prediction of fat oxidation capacity using $^1$H-NMR and LC-MS lipid metabolomic data combined with phenotypic data

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There is evidence from clinical trials that a low capacity to oxidize dietary fats may predispose human individuals to weight gain, obesity, and weight regain following weight loss. These observations have led to a need to identify plasma markers of fat oxidation capacity in order to avoid time consuming direct measurements by indirect calorimetry. The aim of this study was to investigate whether prediction of fat oxidation capacity in an obese population is possible, using $^1$H-NMR and LC-MS-based metabolic profiling of blood plasma samples collected before and after a high fat test meal from 100 obese women, who represented the extremes of fat oxidizing capacity. Subject characteristics (baseline anthropometrics, body composition and dietary records) and clinical data (blood values and derived measures for insulin resistance) were recorded into a phenotypic dataset. Filtering by orthogonal signal correction, variable reduction by spectra segmentation, Mann–Whitney U tests and genetic algorithms were applied to spectral data together with partial least squares regression models for prediction. Our findings suggested that only a small fraction of subject variation contained in metabolic profiles is related to fat oxidation capacity and variable reduction methods improved fat oxidation capacity predictability. The LC-MS dataset led to higher specificity (fasting: 86%; postprandial: 73%) and sensitivity (fasting: 75%; postprandial: 75%) than classification using the $^1$H-NMR dataset (specificity: fasting: 50%; postprandial: 60%; sensitivity: fasting: 67%; postprandial: 62%). Inclusion of phenotypic variables increased specificity and sensitivity values in both fasting and postprandial time points. However, the moderate specificity and sensitivity values indicated that fat oxidation capacity may only be reflected in subtle differences in the metabolic profiles analyzed. In future studies, metabolomics data may be supplemented with gene variation and gene expression data to capture the properties of fat oxidation capacity more precisely.

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1. Introduction

Nowadays, overweight and obesity is a major worldwide issue in the general population. While the link between some biological and behavioral factors, such as the maintenance of a healthy diet, and obesity are known, the molecular determinants associated with obesity are not fully understood. The complex interplay between genes, environment, diet, lifestyle and symbiotic gut microbial activity are among the main factors that affect the health of both individuals and populations, and they may be linked to the mechanisms of fat gain and obesity development [1–3]. Obesity incidence in childhood and its associated risk of developing serious complications such as cardiovascular disease, type 2 diabetes and various types of cancer [4] has led to a need to improve our understanding of the metabolic processes that give rise to abnormal regulation of energy metabolism, which should enable the development of new treatment and prevention strategies.

The current challenge lies in developing early and minimally invasive diagnostic tools to identify people at risk of developing...
obesity, for which a first step may lie in our ability to elucidate the contribution of fat oxidation capacity to the predisposition of subjects to obesity. Indeed, obese people show a limited ability to adjust fat oxidation to their dietary fat intake [5–7]. Additional evidence that the defect in fat oxidation may be primary to the development of obesity, is provided by prospective studies where subjects with low fat oxidation capacity exhibited a greater risk of gaining body weight over time, compared to individuals with high fat oxidation capacity [8,9]. In addition, a number of studies in formerly obese subjects provided evidence of an impaired ability to increase fat oxidation in response to a high fat meal or diet, and to exercise [8,10,11].

The concept of metabolomics was developed on the technical possibility to simultaneously analyze a wide range of metabolites providing metabolic profiles of biological fluids or tissues [12–14]. Through assessing the real end-points of physiological regulatory processes, the metabolites, metabolic profiling enables the measurement of molecular events at various levels of biological organization and provides a systems approach that integrates host and symbiotic partners’ metabolic variations [15]. Such metabolic profiles are most of the time generated by Nuclear Magnetic Resonance (NMR) spectroscopy [14] and Mass Spectrometry (MS) [16,17]. The high density of information contained in metabolic profiles is further explored with statistical methods to recover key information associated to different phenotypes or pathophysiological conditions. Applications of metabolomics in human nutrition and healthcare are associated with its possibilities to define metabolic health and identify early biomarkers of homeostasis disruption. Metabolomics has already demonstrated its potential by characterizing the metabolic effects of nutritional intervention [18,19], effects of the gut microflora on mammalian metabolism [20,21], mechanisms of insulin-resistance [22], disease diagnosis [23] and mechanism of toxicity [24,25].

The present study is based on experimental data generated within the frame of the European multi-center NUGENOB study (www.nugenob.org) whose main objective was to explore the role of interactions between macronutrient composition of the diet and specific genetic variants. A functional fat oxidation capacity test was part of this study, as previously described [5]. These investigations were carried out on two groups of obese women selected from the original NUGENOB study, based on having a high or low increase in fat oxidation following a high fat test-meal, representing the extremes of fat oxidizing capacity. Special focus was paid on the fasting state, since biomarkers present in the fasting plasma could serve as a less invasive diagnostic tool than the ventilated hood system for the assessment of fat oxidation capacity. Indeed, the inherent intra- and inter-individual variability reflected in metabolic profiles of complex organisms can obscure the assessment of metabolic effects due to interventions [19,26–28]. This aspect becomes even more important when considering the limited number of subjects in clinical studies due to practical or budgetary aspects. Supervised learning regression techniques like partial least squares (PLS) have become an often used technique when dealing with datasets that exhibit strong variation and low subject to variable ratio [29–33]. Also, variable filtering methods like orthogonal signal correction (OSC) and variable reduction methods like spectra segmentation, Mann–Whitney U tests and genetic algorithms (GAs) has been applied successfully in previous studies [34–36].

The two main objectives were (i) to compare combination of filtering and regression techniques to identify the most robust and efficient one for biomarker recovery from multivariate metabolic profiles, and (ii) to assess how fat oxidation capacity, a potentially important obesity determinant, could be predicted based on NMR and LC-MS metabolic profiles. Here, we report an exhaustive comparison of different combinations of filtering methods with PLS-regression models and comment on the sensitivity and specificity values obtained for candidate biomarker recovery. Another aim was to assess whether NMR and LC-MS metabolic profiles could enhance prediction of fat oxidation capacity when analyzing them simultaneously with a pre-existing phenotypic dataset.

2. Materials and methods

2.1. Subjects and data

The subjects were a subset of 100 obese women selected from the original NUGENOB cohort of 771 obese subjects (75% women) from eight European centers [37]. Following an overnight fast, the subjects consumed a high-fat test meal [5], which consisted of double cream with 40 g fat/100 g. 95% of the energy in the meal was provided as fat and with 60% as saturated fat [5]. The energy content was fixed at 50% of predicted basal metabolic rate (WHO, technical report series 724, Geneva, 1985). Blood samples were obtained before the test meal and following 1, 2 and 3 h, henceforth referred to as T0, T1, T2 and T3 (See supplementary note for a schematic illustration).

Before, and during the 3 h after the test meal, energy expenditure and substrate oxidation was assessed by ventilated hood (indirect calorimetry). From this the respiratory quotient (RQ), the ratio between carbon dioxide production and oxygen consumption, was estimated. The RQ is an indicator of the ratio between carbohydrate and fat oxidation. The lower the RQ, the higher the fat oxidation [37]. Prior to the test day, a 3-day weighed food record was obtained. The subjects were instructed to keep their habitual diet, and avoid excessive physical activity and alcohol consumption prior to the study. The dietary records were analyzed using the food–nutrient database routinely used in each center [37].

From the 771 subjects, a group of 249 subjects fulfilled the initial selection criteria of being female, having a BMI >29.5 kg/m², having received a test meal corresponding to 40–60% of the measured resting 24 h energy need, having complete measurements of pre and post meal energy expenditure and fat oxidation, having a complete set of biological samples, and finally having completed the subsequent 10-week randomized weight loss intervention. Subjects with fat oxidation measures out of the physiologically plausible range were excluded.

Among these 249 female subjects, the 100 subjects with the highest and the 100 with the lowest capacity for increase in fat oxidation following a high fat test meal were selected. Selection was based on postprandial increase in RQ (as estimated by area under curve) adjusted for age, weight, fat free mass, size of test meal relative to energy need, RQ at fasting state and study center. The residuals from a linear regression model conditioning postprandial increase in RQ on the above-mentioned variables are subsequently referred to as RQRESIDUAL. We constructed RQRESIDUAL in order to use an adjusted RQ measure as a proxy for fat oxidation capacity.

From both groups, samples from 50 subjects were randomly selected for these initial metabolic analyses, and the remaining samples were kept with the possibility of conducting subsequent confirmatory studies. Subjects were not uniformly distributed across all NUGENOB centers, since five centers accounted almost equally for 82 out of the 100 subjects, whereas the last three centers contributed to the resulting 18 subjects (See supplementary tables). Further details about the inclusion and exclusion criteria for the NUGENOB study, experimental design and clinical investigation day as well as information about the sample preparation are reported in literature [5,29,37] and the supplementary materials. Additional metabolomic analyses were conducted in a sub sample of these 100 subjects included in the NUGENOB study. However, these data were not included in the present work as the overall aim was to find biomarkers for fat oxidation capacity in obese individuals and because lean subjects underwent a slightly different selection procedure. In addition, the present study did not include any data from the 10-week low energy diet intervention. The phenotypic dataset included selected data on baseline anthropometrics and body
composition, dietary records, blood values and derived measures for insulin resistance.

The metabolomic profiling, generating 1H-NMR and LC-MS spectral data, was carried out by TNO Quality of Life, Netherlands. Upon collection of blood, plasma was obtained after centrifugation and frozen at −20 °C until analysis. For NMR sample preparation, a volume of 120 µl of blood plasma was added to 530 µl of a deuterated phosphate buffer (0.1 M) solution containing D$_2$O, which was used as a spectrometer field frequency lock. NMR data were acquired on a Bruker AVANCE 600 spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at 600.13 MHz for 1H observation using the standard Bruker Carr–Purcell–Meiboom–Gill (CPMG) spin–echo pulse sequence with water presaturation [38]. The free induction decays (FIDs) were multiplied by an exponential weighing function corresponding to a line broadening of 0.5 Hz prior to Fourier transformation. The acquired spectra were manually phase- and baseline-corrected using the Bruker NMR software, XWINNMR version 3.1 (Bruker Biospin, Rheinstetten, Germany). More details on sample preparation and NMR data generation can be found in reference [29]. Generally, the NMR spectrum enables the simultaneous identification and monitoring of a wide range of endogenous metabolites, thus providing a biochemical fingerprint of an organism [29,39]. Since the CPMG pulse sequence attenuates the signal of macromolecular species, such as proteins and lipids, and contributes to give more insights into the composition of low molecular weight metabolites in the blood plasma, the NMR-based metabolic profiling was designed for assessing metabolic changes not related to lipid composition in blood plasma.

Changes in lipid composition were monitored with the targeted LC-MS approach. For LC-MS sample preparation, 10 µl of blood plasma extract were added to 200 µl of isopropyl alcohol containing 3 internal standards (C17:0 lyso-phosphatidyl choline 1 µg/ml, C24:0 phosphatidyl choline 1 µg/ml and C51:0 triglyceride 2 µg/ml). The samples were centrifuged for 3–5 min at 10,000 rpm to remove the precipitated proteins. The analysis was performed using a Waters HPLC 600 MS pump equipped with a 717 autosampler and a 6005 systems controller (Waters, Etten-Leur, the Netherlands) equipped with a Thermo Finnigan TSQ 700 LX triple quadrupole MS electrospray ionization for mass detection in the positive mode (Thermo, Breda, the Netherlands). For both platforms duplicate measurements were available and the following classes of lipids were analyzed: phosphatidylcholines, lysophosphatidylcholine, diglycerides, triglycerides, sphingomyelins, and cholesterol esters.

2.2. Software

Data processing was performed using Matlab version 7.2.0 (The Mathworks, Natick, MA, USA) and PLS regression was implemented by PLS Toolbox version 3.5 (Eigenvector Research, Wenatchee, WA, USA). The GA was implemented by the Matlab Genetic Algorithm Toolbox version 1.2 (University of Sheffield, Sheffield, UK). Statistical analysis was performed in R version 2.5 [40].

2.3. Strategy for data treatment

The strategy applied for 1H-NMR and LC-MS lipid data preprocessing, data reduction and filtering, and data regression analysis is explained in the following sections (Fig. 1).

2.4. Data preprocessing

Prior to subsequent 1H-NMR data processing, chemical shifts (δ) were aligned by use of the WinLin software version 2.3 (TNO, Zeist, The Netherlands). Next, chemical shifts in the region greater than 4.5 parts per million (ppm) were removed in the NMR-plasma spectra in order to eliminate variation caused by suppression of water resonance and other non-informative variables following the water resonance suppression peak. Elimination of the upper end of the spectra resulted in a little loss of information about aromatic amino acids. Hereafter, cubic spline interpolation was used to extract new data points from the fitted curve to the original data points at regular intervals in order to reduce all spectra from approximately 15,000 variables (chemical shifts) to approximately 7600 variables. Consequently, the intervals of the new spectra were constrained to the ppm region [0 0.4–4.65] with equal step sizes of 0.0005. Then, all spectra were normalized by their total intensity, in order to compensate for differences in concentrations across samples. Prior to averaging of replicates, duplicate measurements were inspected by principal component analysis (PCA) and additionally by assessment of their Pearson correlation coefficients. Since the influence of metabolites occurring at low concentrations was of interest in the present study, auto-scaling (each variable has a mean of zero and a variance of one) was applied.

Prior to data preprocessing the LC-MS lipid dataset consisted of 2287 peak picked variables (m/z values) extracted by an in house developed procedure. Variables with masses below m/z 300 did not represent lipid compounds and were removed from the dataset. As in the NMR dataset, PCA and Pearson correlation coefficients were used to identify and remove outlier samples. Missing value imputation was done by a regular expectation maximization algorithm in Matlab [41]. The LC-MS lipid dataset was auto-scaled.

2.5. Data reduction and filtering

Generally, split-sample procedures or cross validation (CV) approaches are used to assess the predictability and validity of supervised models. Prior to applying variable reduction and data analysis all LC-MS lipid and 1H-NMR time point datasets were split into training and test sets using the ratio 3:1. In the training sets a 5-fold CV procedure was applied to find the optimal number of latent variables (LVs) in the PLS-regression models (Fig. 2). (See supplementary tables for the number of LVs used in each dataset at the various time points.)

We tried various methods to reduce the high-dimensionality of the 1H-NMR and LC-MS datasets in order reduce the risk of over-fitting and fitting to chance-correlations. Different combinations of data filtering and reduction methods were applied to the datasets to find the combination resulting in the highest predictability of fat oxidation capacity based on the test set.

In the NMR dataset the high-dimensionality was reduced by species segmentation. Each spectrum was divided into five regions, each constituting a distinctive ppm-interval with uninterrupted peaks and an approximately equal amount of variables. Spectra segmentation on the one hand reduced the information of each sub-spectra by 80%, on the other hand was it supposed to reduce the high-dimensionality and thereby reduce over-fitting. OSC was used as a filtering method to remove the systematic variation which was not correlated with RQRESIDUAL [36]. In the OSC method 2 LVs and 10-fold cross validation were used.

In the LC-MS dataset a non-parametric Mann–Whitney U test and a genetic algorithm were used to reduce the high-dimensionality. The Mann–Whitney U test was used to identify LC-MS lipid variables that were different between the groups in the training sets. It was preferred over the standard t-test, as the distribution of approximately 25% of the variables were skewed rather than normally distributed (assessed by the Shapiro–Wilk test statistic [42]). Multiple testing corrections was not needed since the Mann–Whitney U test was used for ranking purposes and multiple testing correction does not change the order of the test statistics. Besides the Mann–Whitney U test we assessed how well a GA could select subsets of variables correlated with RQRESIDUAL. Briefly, the GA is a stochastic global search method that implements the idea of natural biological evolution, which employs the principle of survival of the fittest to iteratively produce better solutions of populations of potential solutions (subset of m/z variables)
Fig. 1. Flow chart of the overall data analysis strategy. Each combination of methods employed is illustrated by a path through the diagram.
The fitness function was calculated by a PLS-regression model using 5-fold CV. The parameterization was based on the Grefenstette settings: population 20; probability of crossover 0.9; probability of mutation 0.1; and generations 30 [44]. The GA reduced the spectra from 915 to between 100 and 150 variables for each time point.

2.6. Analysis of phenotypic variables

An explorative PCA analysis was made in order to assess whether fat oxidation capacity can be captured by a linear combination of phenotypes. The underlying assumption was that fat oxidation capacity is correlated with the main part of the variance in the phenotypes. PCA-score plots with 3 LVs were used and samples were color coded according to fat oxidation group.

Linear regression of the RQRESIDUAL against one single phenotype at a time was used to quantify the correlation between the RQRESIDUAL and the various phenotypes.

2.7. Data regression analysis

In the 1H-NMR data regression analyses, the following 3 approaches were assessed: (1) PLS, (2) OSC+PLS, and (3) OSC+PLS on 1H-NMR regions. The metabolic profiles were regressed against the RQRESIDUAL. In the LC-MS analysis we assessed the following combinations of regression models: (1) PLS, (2) OSC+PLS, (3) U-test+OSC+PLS, (3) GA+OSC+PLS, (4) U-test+GA+PLS, and (5) U-test+GA+OSC+PLS. In the simultaneous analysis of variables from the phenotypic dataset and the metabolic profiles, we used the phenotypic variables as additional response variables with RQRESIDUAL. RQRESIDUAL and each phenotypic variable were auto-scaled prior the PLS-regression. One single phenotypic variable was included at a time. This approach was exploratory, and combinations of phenotypic variables were not assessed in the present study.

The predictability of the regression models with and without phenotype inclusion was assessed using sensitivity and specificity as measures. The sensitivity reflects the probability that a subject is predicted as having high fat oxidation capacity given they possess high fat oxidation capacity, whereas the specificity measures the model’s ability to correctly identify subjects with low fat oxidation capacity.

3. Results and discussion

3.1. PLS-regression analysis

Besides the ability to predict fat oxidation capacity we wanted to compare effect of combining different data filtering and selection methods prior to PLS-regression. Both in the NMR and LC-MS dataset we assessed the impact of OSC filtering. Spectra segmentation was assessed as a variable selection method in the NMR dataset, whereas the effect of variable reduction in the LC-MS dataset was assessed by Mann–Whitney U tests and GAs. The different regression models were applied to data at each time point to investigate whether the ability to predict fat oxidation was enhanced during the fasting (T0) or postprandial phase (T1–T3). Overall specificity and sensitivity values were larger in the LC-MS lipid profiles than in the 1H-NMR datasets (Table 1 and Fig. 3). In terms of specificity, time point datasets T0 and T1 exhibited the highest prediction in the LC-MS lipid and 1H-NMR dataset, respectively. In terms of sensitivity, time points T0 and T2 showed the highest prediction of fat oxidation capacity in the 1H-NMR and LC-MS lipid dataset, respectively.

Table 1
Results from 1H-NMR and LC-MS lipid dataset

<table>
<thead>
<tr>
<th></th>
<th>1H-NMR</th>
<th>LC-MS lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLS OSC+PLS OSC+PLS (Regions)</td>
<td>PLS OSC+PLS U-test+OSC+PLS GA+OSC+PLS U-test+GA+PLS U-test+GA+OSC+PLS</td>
</tr>
<tr>
<td>T0</td>
<td>Specificity 0.36 0.29</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Sensitivity 0.33 0.67</td>
<td>0.33</td>
</tr>
<tr>
<td>T1</td>
<td>Specificity 0.47 0.60</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Sensitivity 0.33 0.42</td>
<td>0.50</td>
</tr>
<tr>
<td>T2</td>
<td>Specificity 0.38 0.46</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>Sensitivity 0.62 0.62</td>
<td>0.54</td>
</tr>
<tr>
<td>T3</td>
<td>Specificity 0.36 0.57</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Sensitivity 0.42 0.33</td>
<td>0.42</td>
</tr>
</tbody>
</table>

The highest specificity and sensitivity obtained for each time point are marked with bold.

Fig. 2. Illustration of the splitting into training- and test dataset to assess the prediction of fat oxidation capacity.
Fig. 3. Predicted versus measured RQRESIDUALs in both the $^1$H-NMR and LC-MS lipid test sets at all time points. The $^1$H-NMR panel is based on the OSC+PLS model, whereas the LC-MS panel is based on the $U$-test+GA+PLS regression model. Ideally the relationship between the measured and predicted RQRESIDUAL should have been a straight line through the origin and with a coefficient of one. The deviance from the fitted lines from the theoretically straight line illustrates the relatively low predictability of fat oxidation capacity in the $^1$H-NMR dataset and LC-MS dataset in T3.
3.1.1. Conditional effects of individual filtering techniques on PLS-regression analysis

It is worth noticing that OSC filtering slightly enhanced prediction in both the NMR and LC-MS datasets when compared to models generated without variable filtering techniques. In a next step, we assessed the effect of variable selection methods on the predictability of fat oxidation capacity. Interestingly, division of the 1H-NMR spectra into distinct regions only enhanced sensitivity in time point T1 and specificity in time point T0. The following regions 4.00–4.65; 1.00–2.00; 4.00–4.65; 3.00–4.00 were the most predictive at the time points, T0, T1, T2 and T3, respectively.

Both variable selection by the U-test and GA led to slightly better modeling, when applied separately prior to PLS regression without OSC filtering. By applying the U-test selection we removed variables, which were assumed to be constant across both fat oxidation groups. The U-test variable selection reduced the number of variables from 915 at each time point to 273 (T0), 298 (T1), 302 (T2) and 290 (T3). Combining U-test and GA variable selection increased predictability, showing that the combination of the unsupervised and supervised variable selection method led to a better model, than when using them as standalone variable reduction methods.

3.1.2. Conditional effects of combining filtering techniques prior to PLS-regression analysis

Last, we have assessed the impact of variable selection by U-test and GAs together with OSC filtering. When applying both methods prior to OSC filtering and PLS-regression predictability only increased slightly in T2 compared to the results obtained when applying both methods separately.

Taken together, a combination of unsupervised and supervised variable selection methods may enhance prediction. However, after applying both U-test and GA, only 136 (T0), 144 (T1), 151 (T2), and 154 (T3) variables were retained in the LC-MS dataset. This relative low number of selected variables led to over-fitting when adding OSC filtering prior to PLS-regression, reflected in the increased prediction error on the test set.

The highest predictability of fat oxidation capacity was observed in the LC-MS lipid dataset in the fasting state T0. Two hours after the fat intake we observed the second highest predictability also based on the LC-MS dataset. In both datasets predictability of fat oxidation capacity was decreased in T3 suggesting that alterations in the metabolome contributing statistically to fat oxidation were relatively low 3 h after the high-fat test meal.

3.2. Phenotypic dataset inclusion results

The aim of the inclusion of the phenotypic dataset was to assess whether these variables could enhance the prediction of fat oxidation capacity based on the metabolic profiles. Prior to the PLS-regression analysis we investigated to which extent these variables were correlated with the RQRESIDUAL. The PCA-score plot did not reveal any separation in fat oxidation capacity. This is may be due to the fact that PCA grouped data according to the major variances in the data, which were not correlated with RQRESIDUAL. This was in line with the hypothesis that fat oxidation capacity may only exhibit subtle alterations between high and low fat oxidizers. Additionally, none of phenotypic variables had a correlation coefficient greater than +0.2 or smaller than −0.2, supporting the conclusion from the PCA analysis.

The next step was to assess if inclusion of the phenotypic dataset could enhance prediction of fat oxidation capacity. Due to the low predictability observed in the 1H-NMR datasets, we carried out the phenotype inclusion analysis based only on the LC-MS lipid dataset. Furthermore we chose to use the U-test + GA + PLS approach, since it resulted in the best prediction based on the spectra. The phenotype inclusion analysis was then carried out by adding one phenotype at a time as an additional response variable to the regression model. Interestingly, predictability of fat oxidation increased both in terms of specificity and sensitivity for various phenotypic variables (Table 2). Inclusion of carbohydrate-, protein-, saturated fatty acids- and mono-unsaturated fatty acids dietary records lead to better prediction of fat oxidation capacity in the fasting state. In addition, the fasting blood concentration of insulin-like growth factor increased fasting state predictability. Moreover, inclusion of phenotypic variables also increased predictability in the postprandial state T3, which exhibited decreased prediction without any phenotype inclusion. The phenotypic variables fat and saturated fatty acid dietary records; free fatty acid, triglyceride, insulin and cholesterol fasting blood concentration; total postprandial incremental response of energy expenditure, free fatty acids, triglycerides, cortisol and cholesterol blood concentration increased both specificity and sensitivity. For instance at T3, the inclusion of postprandial incremental response of cortisol blood concentration led to specificity and sensitivity values of 0.82 and 0.57, compared to the specificity and sensitivity values of 0.73 and 0.43 obtained in the regression analysis solely based on the spectra.

3.3. Strengths and limitations

A major strength of this study is the inclusion of a large number of well characterized and standardized subjects and the assessment of the metabolic profile before and after a standardized functional fat oxidation capacity test by means of an oral high fat load test meal. The two groups were selected based on the post meal fat oxidation adjusted for fasting fat oxidation and other relevant measures. This design allowed us to address whether components of the baseline metabolomic profile predicted the response to the high fat test meal, as well as whether the post meal metabolic profile differed between the two groups showing marked differences in the concomitant fat oxidation. Moreover, we demonstrate a new approach in which inclusion of selected phenotypic data as additional response variables in the PLS-regression analysis enhance prediction. The increased predictability obtained by phenotypic variable inclusion in T3, is biologically reasonable, since the differences in fat oxidation capacity between high and low fat oxidizers may become less pronounced during the postprandial time course, as the excess of dietary fatty acids is metabolized. These variables might have counteracted the decrease in detectable alterations in the metabolome in T3.

According to the overall study design, the two groups represented large differences in fat oxidation in response to a high fat test meal. However, our experimental and computational framework had limitations, which may have resulted in the detection of only subtle differences in the metabolic profiles between these groups. These differences were to a greater extent captured in the LC-MS lipid dataset, whereas they were not detectable in the 1H-NMR dataset. Since CPMG spectral data showed attenuated contribution of plasma lipids to the overall profile, whilst LC-MS was targeted to measure specific classes of lipids, our results indicate that very little information contained in the profiles of low molecular weights metabolites could be ascribed to fat oxidation capacity. Thus, these seemingly limited differences in metabolic profiles may lead to a weak prediction. A key question regarding the validity of our approach is whether plasma metabolites closely reflecting fat oxidation capacity would be captured by the platforms used to acquire the metabolomic datasets. Our findings suggest that 1H-NMR and LC-MS lipid may be supplemented by acquiring other NMR sequences such as the standard and diffusion-edited spectra, as well as gas chromatography-mass spectrometry. A reason for the low predictability of the NMR data could be due to the fact that only the CPMG sequence was acquired in this study. The standard and diffusion-edited sequences do not attenuate the signals of lipids and especially lipoprotein signals, which might be of first interest for assessing metabolic changes in lipid mobilization and metabolism. It could be of scientific relevance to acquire the whole panel of spectra (standard, CPMG and diffusion-
Leptin fasting blood concentration Specificity 0.60
Sensitivity 0.67

Cortisol fasting blood concentration Specificity 0.67
Sensitivity 0.60

Insulin fasting blood concentration Specificity 0.60
Sensitivity 0.60

Glucose blood concentration Specificity 0.60
Sensitivity 0.60

Cholesterol fasting blood concentration Specificity 0.60
Sensitivity 0.60

Low density lipoprotein fasting blood concentration Specificity 0.73
Sensitivity 0.50

High density lipoprotein fasting blood concentration Specificity 0.67
Sensitivity 0.67

Insulin like growth factor fasting blood concentration Specificity 0.67
Sensitivity 0.67

HOMA secretion Specificity 0.53
Sensitivity 0.58

HOMA resistance Specificity 0.58
Sensitivity 0.67

Postprandial incremental response of energy expenditure Specificity 0.73
Sensitivity 0.60

Postprandial incremental response of glucose blood concentration Specificity 0.60
Sensitivity 0.47

Postprandial incremental response of free fatty acid blood concentration Specificity 0.60
Sensitivity 0.67

Postprandial incremental response of triglyceride blood concentration Specificity 0.67
Sensitivity 0.53

Postprandial incremental response of insulin blood concentration Specificity 0.60
Sensitivity 0.67

Postprandial incremental response of cortisol blood concentration Specificity 0.79
Sensitivity 0.64

Postprandial incremental response of cholesterol blood concentration Specificity 0.60
Sensitivity 0.73

The U-test + GA + PLS model was used. Specificity and sensitivity values lower than the values obtained without phenotype inclusion are marked in bold. Some phenotypic variables only existed for the fasting or postprandial state. *HOMA secretion = 20×fasting insulin (µU/ml)/(fasting glucose [mmol]/l) × 3.5). **HOMA resistance = (fasting insulin×fasting glucose)/22.5.

edited) on the samples which would give a complete NMR profile for further data analysis. Despite the relatively large sample size compared to other metabolomic studies, another limitation is whether a single split into training and test sets allows a general assessment of the datasets. Changing the training and test sets may result in alterations in specificity and sensitivity. In the test set, subjects were selected from all centers to reflect as closely as possible the overall subject-center distribution. The heterogeneity in the cohort resulting from various environmental factors and the large number of centers participating in the recruitment of subjects may have added non-negligible variance to the analysis. Finally, it is possible those static plasma markers simply do not reflect the tissue specific disturbances in fatty acid oxidation and that either measures reflecting fatty acid dynamics or tissue specific biomarkers are required.

Besides the choice of metabolomic platforms, another limitation might have been the use of PLS regression for prediction. Since PLS regression is a linear model which cannot capture non-linear relationships between RQRESIDUAL and data, other non-linear models may be used in the prediction of fat oxidation capacity. Non-linear PLS, or machine learning tools like random forest regression [45], Lasso
Acknowledgements

We show that fasting metabolomic differences in the LC-MS lipid dataset are more pronounced than postprandial metabolomic differences and that inclusion of selected phenotypic variables may enhance both fasting and postprandial predictability.

In future studies, complementing techniques may be used to investigate other parts of the metabolome that were not captured in the datasets of the present study. In addition, modeling of fat oxidation capacity by metabolomic and phenotypic data, may be supplemented by gene variation and gene expression data in order to increase power in the statistical analysis. For optimizing study design and data modeling, future studies are expected to benefit from considering relevant phenotypic variables which may be obtained for the purpose of refining the prediction models/statistical models, as well as considering the strengths of a design including a baseline measurement followed by measurements during the subject to a standardized intervention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.chemolab.2008.03.008.

References


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