

A predictive metabolomics evaluation of nutrition-modulated metabolic stress responses in human blood serum during the early recovery phase of strenuous physical exercise

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6 1 ***A predictive metabolomics evaluation of nutrition-modulated***
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9 2 ***metabolic stress responses in human blood serum during the***
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11 3 ***early recovery phase of strenuous physical exercise***
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4 19 **Abstract**
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7 20 We have investigated whether post-exercise ingestion of carbohydrates in combination with proteins
8 21 generates a different systemic metabolic response, as compared to the sole ingestion of carbohydrate or
9 22 water, in the early recovery phase following exercise. In addition, metabolic patterns related to fitness level
10 23 were studied together with individual responses to nutritional modulation. Twenty four male subjects were
11 24 exposed to 90 min of ergometer-cycling. Each participant was subject to four identical test-sessions, including
12 25 ingestion of one of four beverages (water, low carbohydrate beverage, high carbohydrate beverage and low
13 26 carbohydrate-protein beverage (LCHO-P)) immediately after cycling. Blood was collected at six time points, one
14 27 pre- and five post-exercise. Extracted blood serum was subject to metabolomic characterization by gas
15 28 chromatography / time of flight mass spectrometry (GC/TOFMS). Data was processed using hierarchical
16 29 multivariate curve resolution (HMCR), and multivariate statistical analysis was carried out using orthogonal
17 30 partial least squares (OPLS). Predictive metabolomics, including predictive HMCR and OPLS classification, was
18 31 applied to ensure efficient sample processing and validation of detected metabolic patterns.
19 32 Separation of subjects in relation to ingested beverage was detected and interpreted. Pseudouridine was
20 33 suggested as a novel marker for pro-anabolic effect following LCHO-P ingestion, which was supported by the
21 34 detected decrease of the catabolic marker 3-methylhistidine. Separation of subjects according to fitness level
22 35 was achieved and nutritional modulation by LCHO-P was shown to improve the metabolic status of less fit
23 36 subjects in the recovery phase. In addition, the potential of the methodology for detection of early signs of
24 37 insulin resistance was also demonstrated.

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36 38 **Keywords:** GC-MS, metabolomics, predictive metabolomics, chemometrics, human, exercise, serum, recovery,
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42 ***Introduction***

43 To assess the wealth of metabolic interactions occurring in humans induced by complex nutrients,
44 nutritional and food scientists have started to appreciate the value of the 'omics' technologies in combination
45 with advanced multivariate data analysis and interpretation tools.¹ It is reasonable to assume that the
46 introduction of a nutrient or food containing hundreds of compounds to an already complex human metabolic
47 system, will require tools that can detect and provide an interpretation of the simultaneous interplay between
48 a large number of genes, proteins or metabolites in order to get an enhanced understanding of the effects. So
49 when introducing additional stressors, e.g. disease or exercise, the problem becomes even more complex. This
50 does, in our mind, even further emphasise the need for utilizing this type of multivariate profiling techniques.
51 Not many studies to date have applied multivariate 'omics' profiling to investigate the complex metabolic
52 responses caused by the interaction between physical exercise and nutrition, which implies that this is an area
53 of great future interest.

54 The level and type of hormonal and cell-signaling events during exercise and in the early recovery phase
55 are likely influenced by the amplitude of diverse systemic and intracellular metabolic responses, related to
56 exercise workload and muscles energy homeostasis. These stress-induced responses may lead to altered gene
57 and protein expressions and, ultimately, an adapted phenotype to increased workload and performance with
58 training (days-weeks-months). Related to this, a growing number of studies clearly show that gene expression
59 in the recovery phase from exercise can be modulated by nutritional intake²⁻⁷. Thus, there is a growing interest
60 to study the physiological interactions between exercise metabolism and intake of macronutrients, during
61 training. So far this has concerned mainly carbohydrates and proteins, as these have been suggested to play a
62 significant role for the adaptive phenotype to progressive workloads throughout periods of training and,
63 ultimately, improved performance⁸⁻¹⁰. Importantly, the level and the activity of regulative factors that
64 influencing the pro-anabolic versus pro-catabolic responses in the early recovery phase (within the first hours)
65 from exercise will probably have a significant impact on the outcome of training. Co-ingestion of carbohydrates
66 and proteins generates an enhanced insulinotropic effect following exercise compared with carbohydrates
67 alone, which is associated with a superior glycogen resynthesis in skeletal muscle¹¹. However,
68 hyperinsulinaemia has been suggested to play a greater role in the slow, second phase of the glycogen
69 resynthesis than in the initial phase following exercise¹². Since insulin promotes cell-signaling events¹³, gene

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3 70 expression¹⁴, uptake of amino acids, protein synthesis, and reduced protein degradation in skeletal muscle¹⁵,
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5 71 the major role of hyperinsulinaemia in the early recovery phase following exercise is likely to be attributed to
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7 72 the anti-catabolic and pro-anabolic effects in muscle. Nevertheless, increased levels of glucose, amino acids
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9 73 and, consequently, hyperinsulinaemia are together likely to be a major determinant of muscle recovery and
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11 74 adaptation, which suggest that investigation of such metabolic interactions on a global level could provide new
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13 75 insights and generate novel hypotheses regarding nutritional modulation effects on human physiology.

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16 76 Metabolomics^{16,17} can be seen as a tool for global metabolite analysis that is now starting to prove itself as
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18 77 an important player in systems biology¹⁸. In essence, the idea is to characterize metabolomes in biofluids, cells,
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20 78 tissues, and organisms by simultaneous quantification of all detectable small molecules and to monitor the
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22 79 systematic pattern changes therein induced by e.g. genetic modification¹⁹, exogenous compounds²⁰, or
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24 80 disease^{21, 22}. Recently the added value of metabolomics for advancing the area of clinical nutrition towards
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26 81 personalized health has been discussed²³. The metabolomic characterization is done by using sensitive
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28 82 analytical techniques, where nuclear magnetic resonance spectroscopy (NMR), gas chromatography/mass
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30 83 spectrometry (GC/MS), and liquid chromatography/mass spectrometry (LC/MS) are the most commonly
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32 84 applied. Ultimately the end goal is to detect single metabolites or patterns of metabolites indicative of a
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34 85 specific physiological state; so-called biomarkers or biomarker patterns, or to get a deeper understanding of
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36 86 complex metabolic interactions in relation to this stated physiology. To reach these goals, reliable comparisons
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38 87 between multiple samples based on the whole set of variables (i.e. hundreds to thousands of quantified
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40 88 metabolites) is a prerequisite.

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44 89 Predictive metabolomics²⁴ is a concept combining metabolomics with chemometrics^{25, 26} for generating
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46 90 representative data²⁷ and for performing multivariate sample comparisons to obtain reliable biomarker
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48 91 detection, interpretation, prediction/diagnosis and validation. In addition, the predictive metabolomics
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50 92 approach allows screening of large sample sets in a high-throughput fashion without compromising the data
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52 93 quality²⁸. Recently we applied the approach for investigating the acute effects of strenuous physical exercise in
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54 94 human blood serum²⁹. Our results were very encouraging since they verified that we could detect exercise
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56 95 related systematic changes in a large number of identified or identifiable metabolites in human serum. In
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58 96 addition we could interpret these metabolic interactions, and importantly make predictions of the metabolic
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60 97 status in humans on an individual basis.

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3 98 The objective of the present study was to investigate whether post-exercise ingestion of carbohydrates in
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5 99 combination with proteins generates a different systemic human blood serum metabolic response, compared
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7 100 to the sole ingestion of carbohydrates or water, in the early recovery phase following exercise. In addition,
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9 101 metabolic patterns related to subjects' fitness level was investigated together with the individual response to
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11 102 nutritional modulation in the early recovery phase for participating subjects with the lowest fitness level
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13 103 (LowFit). To address these issues we here utilize a predictive metabolomics protocol including a hypothesis-free
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15 104 metabolomic screening approach of human blood serum using gas chromatography/time-of-flight mass
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17 105 spectrometry (GC/TOFMS) combined with hierarchical multivariate curve resolution (HMCR)³⁰, and
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19 106 chemometric data evaluation by means of orthogonal partial least squares (OPLS)³¹. The proposed
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21 107 methodology can be seen as a tool for interpreting complex interaction patterns reflecting human exercise and
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23 108 nutritional metabolism as well as metabolic disorders.
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111 **Methods**

112 **Study population and experimental procedures**

113 Twenty four healthy males involved in regularly exercise training, with a mean (\pm SD) age of 25.7 \pm 2.7 years,
114 height 182.5 \pm 7.6 cm, body weight 77.4 \pm 8.8 kg and with an average peak oxygen uptake (VO₂peak) of 59.1 \pm 7.3
115 mL kg⁻¹ min⁻¹, volunteered to participate in the study after given a signed informed written consent. The study
116 was approved by the regional ethical committee of medical research (# 05-069M). Briefly, each subject
117 participated in a pre-experimental ergometer-cycling VO₂peak test and four identical experimental tests, which
118 consisted of ingestion of one beverage immediately after 90 min standardized ergometer-cycling, with an
119 interval of 1 week between each test occasion. The intervention included ingestion of one of four beverages
120 (different in macronutrient composition) immediately after completion of the 90 min ergometer cycling. Each
121 subject completed all four tests in a randomized order, ingesting one beverage per test.

122 Beverage No 1 was 5 dl tap water (Water), No 2, low carbohydrate beverage (LCHO), contained 1 gram
123 carbohydrates per kilogram body weight in tap water (16% w/v), No 3, high carbohydrate beverage (HCHO),
124 contained 1.5 gram carbohydrates per kilogram body weight in tap water (24% w/v) and No 4, low
125 carbohydrate-protein beverage (LCHO-P), contained 1 gram carbohydrates per kilogram body weight in tap
126 water (16% w/v) and 0.5 gram protein per kilogram body weight. Beverage No 4 was a commercially available
127 recovery beverage (Gainomax recovery, Norrmejerier, Umeå, Sweden) and beverage No 2 and No 3 were
128 beverages prepared at our lab with matching carbohydrate composition as beverage No 4. The protein source
129 of beverage no 4 was milk protein in a ratio of 90% casein and 10% whey. The composition of carbohydrates in
130 beverages 2-4 was 37.5% maltodextrin, 31.25% saccharose, 15.6% glucose and 15.6% galactose.

131 A standardized breakfast was ingested at 7.30 am, one hour prior to each experimental test. The breakfast
132 consisted of drinkable yoghurt in an amount related to the subjects' bodyweight (0.5g carbohydrate/kg
133 bodyweight). The amount of carbohydrates, protein and fat in the yoghurt were 10, 3 and 0.5 percentage by
134 weight of yoghurt respectively. Subjects were instructed to maintain food diaries the day prior to test 1 and
135 then repeat the same diet prior to test 2, 3 and 4. Subjects were also instructed not to perform any exercise or
136 consume alcohol the day before each test and to avoid stress in the morning of the test day.

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3 137 The subjects arrived at the laboratory at 8:00 a.m. and signed a health form. Venous blood samples were
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5 138 taken after 15 min of bed rest (pre-exercise samples) using a vacutainer system (Becton Dickinson, U.K.).
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7 139 Thereafter, subjects were equipped with an intravenous catheter (Optiva2, Medex) in a superficial forearm
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9 140 vein. Subjects then performed 90 min of ergometer-cycling, using an electronically braked bicycle (Rodby, RE
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11 141 829, Vänge, Sweden). Each 90 min test-session consisted of nine equal 10 min sections without rest. The
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13 142 workloads during the sections corresponded to 40% (2 min), 60% (6 min), and 85% (2 min) of the ergometer-
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15 143 cycling VO₂peak work-load at the pre-experimental test. Subjects were asked to maintain a steady cadence of
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17 144 80 rpm during the whole session of each experimental test, and after every 10 min of cycling, 100 mL of water
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19 145 was ingested as well as at +15, +30 and +60 minutes post exercise (after blood collection). Immediately after,
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21 146 and at +15, +30, +60 and +90 minutes after completed ergometer cycling, blood samples were collected from
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23 147 the vein catheter into vacutainer tubes (post-exercise samples). Serum was extracted from the collected blood
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25 148 samples following 8 min centrifugation (+4°C at 3000g) and immediately frozen and stored in -80 °C until time
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27 149 of analysis. Routine methods at the Department of Clinical Chemistry, a certified laboratory at the University
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29 150 Hospital of Uppsala, were used to determine the concentrations of glucose, free fatty acids (FFA) and insulin.
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33 151 **Metabolic profiling**

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35 152 Prior to GC/TOFMS analysis the serum samples were extracted and derivatized according to A et al³². The
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37 153 samples were then injected in splitless mode by an Agilent 7683 autosampler (Agilent, Atlanta, GA, USA) into
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39 154 an Agilent 6890 gas chromatograph equipped with a 10 m x 0.18 mm i.d. fused silica capillary column with a
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41 155 chemically bonded 0.18 µm DB 5-MS stationary phase (J&W Scientific, Folsom, CA, USA). The column effluent
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43 156 was introduced into the ion source of a Pegasus III time-of-flight mass spectrometer, GC/TOFMS (Leco Corp., St
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45 157 Joseph, MI, USA). A more detailed description of the sample preparation, derivatization and GC/TOFMS
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47 158 protocol can be found in the supplementary information.
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51 159 **Sample selection**

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53 160 GC/TOFMS analysis was performed over two runs separated by eight months. Run1 included all serum
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55 161 samples from subjects given water or low carbohydrate-protein (LCHO-P) post exercise. Run2 consisted of all
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57 162 serum samples from subjects given low or high carbohydrate beverages immediately after completing the
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59 163 exercise protocol. The analytical run order in each of the two runs was based on a subset of samples spanning
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164 the principal component (PC) space calculated from physiological property data variables, e.g. age, body mass

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3 165 index and VO₂peak, according to Thysell et al²⁷. A training set for hierarchical multivariate curve resolution
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5 166 (HMCR)³⁰ was selected using a space filling design³³ in PC space calculated from GC/TOFMS data processed
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7 167 using hierarchical multivariate data compression³⁴ (a fast processing method not involving curve resolution)
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9 168 together with physical property data from pre exercise serum samples. By basing the selection on pre exercise
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11 169 samples the best coverage of the normal physiological variation among the samples will be included in the
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13 170 training set. Thus, the selected pre exercise samples (training set), with corresponding recovery samples, were
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15 171 subject to HMCR with optimized settings²⁷. The remaining unprocessed samples were then predictively
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17 172 resolved using the HMCR parameters obtained when resolving the training set²⁴.

173 Data treatment

174 GC/MS analysis of serum samples will produce both overlapping chromatographic profiles and mass
175 spectra, from compounds not separated in the chromatographic dimension. This leads to difficulties regarding
176 metabolite detection, quantification and identification. Therefore a data processing method involving curve
177 resolution was applied to further resolve the detected compounds.

178 The HMCR method produces a data matrix where all samples are described by a common set of quantitative
179 variables describing the relative concentrations of the resolved compounds (putative metabolites), i.e. the area
180 under the resolved chromatographic profile. In addition, each resolved compound has a corresponding mass
181 spectral profile, stored separately, which can be subject to spectral database comparison or *de novo*
182 identification. The resolved data were normalised by means of 12 added internal standards, eluting over the
183 whole chromatographic time range. First, we calculated the area under the chromatographic peak for all
184 internal standards using unique mass-channels. Secondly, noisy mass channels were discarded and a principal
185 component (PC) was calculated for the remaining internal standard mass-channels (all variables were scaled to
186 unit variance, non-centred). The calculated PC score value for each sample was then used to normalize the
187 resolve data by dividing each sample with the corresponding score value. This normalisation procedure is
188 comparable to dividing each sample with the mean concentration of all internal standards, but with the
189 possibility to inspect sample variability among the internal standard concentrations. All internal standards and
190 their unique mass-channels used in the normalization procedure are listed in Table S1 in the supporting
191 information. The metabolic variation for each subject was corrected by means of the acute serum status, i.e.
192 the concentration in samples taken immediately after exercise was subtracted from samples taken in the

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3 193 recovery period, for each subject. In this way each subject acts like its own control which will allow reliable
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5 194 comparisons of the metabolic alterations between subjects even if they have been analytically characterized at
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7 195 different time points. Additional variation related to subjects daily fitness status will also be accounted for
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9 196 when subtracting the post exercise concentration from the corresponding recovery samples.

12 197 **Statistical Analysis**

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15 198 Principal Components Analysis (PCA)³⁵, here applied when selecting representative samples, reduces a
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17 199 multidimensional space, i.e. a dataset described by a high number of correlated variables, into a low number of
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19 200 orthogonally constrained principal components describing the systematic variation in the original dataset.
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22 201 Multivariate regression analysis in terms of orthogonal partial least squares (OPLS)³¹ and OPLS- discriminant
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24 202 analysis (OPLS-DA)³⁶ was applied to extract and interpret the systematic variation in the resolved GC/TOFMS
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26 203 serum profiles related to specific responses. The objective was to extract metabolic patterns in the early
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28 204 recovery phase related to the ingestion of different beverages as well as to clinical measurements of insulin,
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30 205 glucose and fatty acid concentrations. OPLS has not only the ability to separate systematic variation from noise
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32 206 in a dataset but also to further divide it into response related variation and variation orthogonal to the
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34 207 response. Thus, interpretation of complex biological interactions in large omics datasets and the detection of
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36 208 biomarkers or biomarker patterns are facilitated. A schematic picture of the multivariate statistical analysis
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38 209 rationale is presented in **figure 1**.

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42 210 Validation was carried out to evaluate the robustness and reliability of both the analytical (GC/TOFMS) and
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44 211 the mathematical (curve resolution and multivariate regression) procedures. This included running analytical
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46 212 replicates of samples that were used to evaluate the quality the data processing carried out by predictive
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48 213 HMCR (data not shown). Sevenfold full cross-validation with randomized cross-validation groups³⁷ were used to
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50 214 decide the model complexity. Predictions of independent samples for the regression models were performed
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52 215 for validation of the predictive ability of the calculated models and pair-wise t-tests for employed examining
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54 216 the significance of highlighted metabolites (biomarkers).

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58 217 Visualization of the multivariate models was achieved by plotting the extracted latent variables and varied
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60 218 depending on the purpose of the model. To reveal and interpret class separations the cross-validated OPLS-DA
219 scores were used for visualization of the inter subject variation, while the corresponding OPLS-DA loadings

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3 220 were used to provide maps of all resolved metabolites in relation to the inter subject variation. The relationship
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5 221 between the resolved metabolites and the results from measurements of insulin, glucose and free fatty acids
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7 222 (FFA) was visualized by plotting the OPLS loadings.
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10 223 **Identification of metabolites**

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12 224 Resolved metabolite profiles were identified by means of a spectral database search using NIST MS-Search
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14 225 v. 2.0³⁸. Match values ranking the spectra were calculated using the dot product of the two spectra (i.e. the
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16 226 resolved spectrum and the database spectrum), with higher m/z peaks having more weight than lower m/z
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18 227 peaks, since higher m/z values are considered to be more compound specific. Furthermore, a reverse logic
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20 228 which ignores "impurity" (i.e. non-matching) peaks in the resolved spectrum was performed. This reverse
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22 229 match factor is not penalized for peaks in the target spectrum that are not present in the database spectrum.
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25 230 The match values have a range from 0-999, where 999 indicates an identical match. Positive identification was
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27 231 performed by combining match values with retention time index, calculated from the analytically characterized
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29 232 alkane series (C₁₂-C₃₂).
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234 **Results**

235 **Data treatment**

236 In total 576 serum samples were analyzed by GC/TOFMS in two analytical runs. Each GC/TOFMS run
237 consisted of 288 samples, including all subjects and time points related to the ingestion of two different
238 beverages. Prior to curve resolution 17 samples were excluded due to low analytical quality. A training set of 95
239 samples was subject to HMCR. Alignment and smoothing using a moving average was performed prior to
240 dividing the chromatogram into 68 time windows from which a total of 218 chromatographic profiles (peaks,
241 i.e. putative derivatized metabolites) with corresponding mass spectrum were resolved. The remaining 464
242 samples were predictively resolved according to the model HMCR parameters, i.e. resolving matching peaks. By
243 subtracting the peak area (corresponding to relative metabolite concentration) immediately after performed
244 exercise (time point zero) from corresponding recovery samples, a normalization was done to reduce run order
245 effects as well as the daily variation in each subjects' fitness level. This resulted in 453 normalized samples
246 describing the recovery period. Prior to multivariate sample comparison modelling a manual mass spectral
247 filtration was performed to exclude internal standards and peaks with low quality mass spectra. In total 189
248 potential metabolites, from which 53 were identified with high certainty, were retrieved and used in further
249 multivariate sample comparison modelling. The 136 'unidentified' metabolites, also included in the analysis,
250 still retain information regarding their chemical properties, e.g. fragmentation pattern and retention time,
251 which could be used to further classify these compounds. Information regarding the identified metabolites, e.g.
252 retention time precision, match values and abbreviation explanations is presented in **table 1**.

253 **General effects on glucose, insulin and free fatty acids in relation to macronutrient** 254 **ingestion following exercise**

255 As expected, an intake of low (LCHO) and high carbohydrate (HCHO) and carbohydrate-protein beverage
256 (LCHO-P) following exercise resulted in a macronutrient related elevation of insulin and glucose compared to
257 the sole ingestion of water ($p < 0.001$). Additionally, an apparent decline of free fatty acids (FFA), occurring
258 15min after macronutrient intake, was seen. The constant high concentration of FFA throughout the recovery
259 period in subjects ingesting water indicates a continuous catabolic state compared to the FFA response in
260 subjects ingesting protein and/or carbohydrates ($p < 0.001$). A number of amino acids released in the

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3 261 bloodstream in response to protein ingestion , e.g. leucine, promote insulin secretion from the pancreatic beta
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5 262 cells³⁹ and could thereby explain the significantly higher insulin concentration when ingesting LCHO-P
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7 263 compared to LCHO beverage (p<0.001), not observed when ingesting HCHO. Moreover, the increase in glucose
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9 264 when ingesting LCHO and HCHO beverage compared to LCHO-P (p<0.001) could therefore be a result from an
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11 265 increased uptake of glucose in the cell, triggered by insulin. The observed macro nutritional response of insulin,
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13 266 FFA, and glucose is presented as mean values of the resolved relative concentration in **figure 2**. These results
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15 267 suggest differently obtained metabolic responses in relation to the four beverages. However, these important
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17 268 physiological parameters provide only a limited view of the complex metabolic response to macronutrients in
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19 269 humans. Additional multivariate investigation of all extracted metabolites, i.e. the 189 resolved profiles, could
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21 270 therefore provide a more detailed description of the metabolic response from different nutritional intake.
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23 271 Interestingly, two subjects revealed a deviating insulin response, when ingesting carbohydrate containing
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25 272 beverage following exercise, which could indicate an early developed insulin resistance. The two deviating
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27 273 subjects were therefore not included when calculating the arithmetic mean for the insulin concentration.
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274 **General metabolomic recovery response in relation macronutrient addition following** 275 **exercise**

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35 276 Only 9.48% of the variation in the metabolite data was used to describe a large proportion of the metabolic
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37 277 variation which was captured by glucose, insulin and FFA in an OPLS model, leaving a considerable amount of
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39 278 metabolite information left to investigate. Thus, an OPLS-discriminant analysis (OPLS-DA) model was calculated
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41 279 to create a specific overview of the changes in metabolites and metabolite patterns related to the ingestion of
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43 280 different beverages. The resulting score plot (map over samples) revealed a nutrition dependent separation of
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45 281 subjects from all time points in the recovery period, defined mainly by the first two predictive components
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47 282 (**Figure 3A**). The third predictive component separated subjects ingesting the two different carbohydrate
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49 283 beverages (not shown). The corresponding loading plot (map over metabolites) provided detailed information
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51 284 regarding different metabolites and classes of metabolites responsible for the observed nutrition dependent
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53 285 separation of subjects (**Figure 3B**). In general, amino acids (increased after ingesting LCHO-P), fatty acids
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55 286 (increased when ingesting water) and sugars (increased after ingesting LCHO or HCHO) are responsible for the
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57 287 main separation between subjects ingesting different beverages. To investigate the influence of the added
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59 288 amino acids *via* the LCHO-P beverage, an OPLS-DA model was calculated with the amino acid peaks excluded.
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3 289 The metabolic pattern revealed by the model with the amino acid peaks excluded was equivalent to the
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5 290 pattern obtained with the amino acids included, i.e. matching metabolites were responsible for the separation
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7 291 between subjects in relation to ingested beverage. More specifically, subjects ingesting LCHO-P showed a
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9 292 decrease in 3-methylhistidine (3-MeHis), a urinary marker for protein (myofibrillar) breakdown and an increase
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11 293 in pseudouridine (PSU), a modified nucleoside, compared to the sole ingestion of carbohydrate or water. In
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13 294 addition, the ingestion of LCHO-P produced an increase in cholesterol (Chol) and 4-deoxyerythronic acid (4-
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15 295 DEA). Other interesting findings were metabolites which deviated from their general class response, i.e. suberic
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17 296 acid (SuA), a dicarboxylic fatty acid, and glycine (Gly), a common amino acid which acts as an inhibitory
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19 297 neurotransmitter⁴⁰. SuA did not increase after ingesting water, compared to the ingestion of macronutrients, in
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21 298 comparison with other fatty acids, e.g. arachidonic acid (AA) and linoleic acid (LA). In addition the Gly
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23 299 concentration, as compared to general amino acids, decreased when ingesting carbohydrate beverages, i.e.
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26 300 LCHO and HCHO, which was not observed when ingesting water and LCHO-P.
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29 301 **Dynamic metabolomic recovery response of macronutrients in the early recovery** 30 31 **period following exercise** 32

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34 303 To further investigate the dynamics of the metabolic responses, separate time point specific OPLS-DA
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36 304 models were calculated comparing all beverages with each other. The metabolic response was altered at all
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38 305 time points following ingestion of macronutrients, compared to water, with the exception of 15min into the
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40 306 recovery period after intake of LCHO. Additional alterations of the metabolic response were also achieved
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42 307 when ingesting LCHO-P compared to HCHO (caloric match) and the LCHO (equal amount of carbohydrates). No
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44 308 time point specific difference in the metabolic response was found when comparing high and low CHO intake;
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46 309 differences could only be observed when including all time points. From this, dynamic nutritional responses of
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48 310 individual metabolic entities were easily extracted and interpreted. Overall, the amino acid concentration
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50 311 remained elevated for subjects ingesting LCHO-P throughout the whole recovery period, i.e. 90min, compared
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52 312 to the ingestion of water and carbohydrates. The general dynamic metabolic response of fatty acids was more
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54 313 diverse. At 30min after intake fatty acid concentrations were slightly elevated for subjects ingesting LCHO-P
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56 314 followed by a sharp increase in subjects ingesting water for the rest of the recovery period. In more detail, the
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58 315 above mentioned muscle catabolic marker 3-MeHis revealed a sharp decrease in concentration when ingesting
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60 316 LCHO-P beverage at 15 and 30min in recovery ($p < 0.001$, comparing LCHO-P intake to water, LCHO and HCHO at

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3 317 15 and 30min after exercise). After 60min the effect from LCHO-P intake on 3-MeHis was reduced and 90min
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5 318 after intake the effect was gone (**Figure 4A**). The increase in serum concentration of PSU, chol and 4-DEA when
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7 319 ingesting LCHO-P showed a somewhat different response dynamics. The modified nucleoside PSU peaked
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9 320 30min after intake ($p < 0.001$, comparing LCHO-P intake to LCHO and HCHO and p -value: 0.09 compared to water
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11 321 at 30min after exercise) and no significant effect was seen after 60min (**Figure 4B**), while the increase of Chol
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13 322 and 4-DEA lasted until 90min after intake when there was no difference between the beverages. Additional
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15 323 interesting macronutrient responses from a range of metabolites, both identified and unidentified, were
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17 324 observed, not all are discussed here. Both the depletion of Gly for subjects ingesting carbohydrates and the
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19 325 observed non-response of SuA, i.e. did not increase in subjects ingesting water, were stable during the
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21 326 investigated 90min after intake. A thorough validation of the calculated OPLS-DA models was performed by
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23 327 means of independent sample predictions (model specificity) and a sevenfold cross validation (model
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25 328 selectivity). All information concerning the calculated regression models is presented in **table 2**.

29 329 **Metabolomic recovery response in relation to subjects fitness status and** 30 31 330 **macronutrient modulation**

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33 331 A different recovery response was observed in relation to fitness level, here defined by the oxygen uptake
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35 332 (VO_{2peak}) established in a pre-test. Subjects with the top five VO_{2peak} ($68.2 \pm 2.9 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) were
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37 333 defined as 'HighFit' subjects and compared to subjects with the bottom five VO_{2peak} ($49.4 \pm 6.4 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$),
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39 334 here defined as 'LowFit' subjects. An OPLS-DA model was calculated to classify the selected subjects
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41 335 according to fitness level. The model was based on each subjects' recovery period after ingesting water only.
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43 336 The cross-validated OPLS-DA scores (**Figure 5A**) revealed a clear pattern, separating each individual based on
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45 337 fitness level, and the OPLS-DA loadings (**Figure 5B**) provided a map for interpretation of the metabolic
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47 338 differences related to this difference. Overall, there was a general increase of both amino and fatty acids in
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49 339 HighFit subjects. More specifically, arachidonic acid (AA) levels, an essential fatty acid and precursor of
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51 340 eicosanoids⁴¹, was elevated in HighFit subjects compared to LowFit, which was also the case for the modified
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53 341 nucleoside and potential marker for increased protein synthesis, pseudouridine (PSU). SuA, which has been
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55 342 reported to increase with age and high β -oxidation activity^{42, 43}, was elevated in the LowFit subjects together
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57 343 with creatinine (Cr), cholic acid (CA) and cholesterol. This result suggests that we potentially could make
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59 344 predictions of, monitor and interpret physiological status in humans on an individual basis. Furthermore, the
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3 345 OPLS-DA model separating subjects according to fitness state was used to predict the metabolic status of the
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5 346 LowFit subjects following LCHO-P intake. Interestingly we can confirm from the cross-validated OPLS-DA scores
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7 347 (Figure 5A) that all the individual subjects are moving in the direction of the HighFit subjects, when ingesting
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9 348 macronutrients, and the magnitude of the movement can be followed on an individual basis. This implies that
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11 349 we possibly have an instrument for individualised modulation that can be used for i) optimising recovery and
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13 350 physical fitness or ii) preventing, reversing or even treating metabolic disorders, on an individual basis. As can
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15 351 be observed in the OPLS-DA scores (**Figure 5A**) one subject is clearly showing an abnormal behaviour at two
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17 352 time points, i.e. 15 and 90min after LCHO-P intake. Coincidentally, this was one of the subjects displaying an
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19 353 abnormal increase serum insulin levels at 15-30min after completing exercise with LCHO-P intake (Figure 5A,
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21 354 inserted). One interesting finding in the metabolomic data for this particular subject was a parallel increase in
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23 355 the concentration of myo-inositol. This increase in myo-inositol was also seen in the other subject showing an
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25 356 abnormal insulin response (data not shown).
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367 **Discussion**

368 **Physiological interpretation**

369 Each of the four beverages ingested immediately after exercise produced different systemic patterns
370 related to the difference in macronutrient composition. In general, a metabolic marker pattern of a catabolic
371 state, such as increased level of fatty acids and decreased levels of amino acids and sugars, was observed
372 related to the intake of water in the recovery phase. In contrast, an apparent pattern associated to an anabolic
373 state was detected following intake of LCHO-P. These are all expected responses as the intake of carbohydrates
374 produces both direct effects, such as increased levels of sugars in blood, and indirect effects, exemplified by
375 the insulin response. Accordingly, the elevated level of amino acids following ingestion of LCHO-P can likely
376 explain the enhanced insulin response following ingestion of the LCHO-P beverage, as a number of amino acids
377 promote insulin secretion from the pancreatic beta cells⁴⁰. The increase in insulin level observed following
378 exercise, when a combination of carbohydrates and proteins was ingested, is likely to explain the marked
379 decline of FFA levels detected after 15 minutes in recovery, not observed when ingesting water only. A
380 probable reason for this is the suppressed release of FFA from the adipose tissue, as a result of increased level
381 of insulin and its inhibition of hormone sensitive lipase (HSL) activity that hydrolyzes stored triglycerides to
382 FFA⁴⁴.

383 An interesting finding from the metabolomics data were the elevated pseudouridine (PSU) level detected
384 at 15 and 30 minutes post exercise (**Figure 4B**) as an effect from ingesting LCHO-P. PSU is the C-glycoside
385 isomer of the nucleoside uridine deriving from catabolism of both rRNA and tRNA not salvaged once released
386 from cells. The level of PSU in urine is a known marker of whole-body turnover rate of tRNA and rRNA⁴⁵,
387 correlating to resting metabolic rate and energy intake^{46,47}. In addition, urinary PSU levels have also been used
388 as an indicator of disease states such as cancer⁴⁸ and inflammation⁴⁹. The increased serum PSU levels seen in
389 the early recovery phase from exercise following ingestion LCHO-P is likely to be a consequence of an
390 accelerated protein synthesis, thus increased RNA turnover, in tissues stressed by exercise, particularly the
391 skeletal muscle. Related to this theory, it is recognized that an increase in insulin level induces cell-signaling,
392 gene expression and protein synthesis as well as restraining processes of protein catabolism in human skeletal
393 muscle. Additionally, an increased systemic level of amino acids as a consequence of ingestion of mixtures with
394 proteins/peptides/amino acids affects gene expression^{50,51} and generates anabolic cell-signaling responses, i.e.

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3 395 increased activity of key enzymes in protein synthesis, after physical exercise in human skeletal muscle⁵¹⁻⁵³.
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5 396 Furthermore, increased insulin levels seen following the ingestion of carbohydrates after exercise are triggered
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7 397 by co-ingestion of proteins that increases the level of amino acids⁵⁴. Collectively, the elevated concentration of
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9 398 insulin along with an increased availability of amino acids for protein synthesis may well explain the elevated
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11 399 PSU level. Thereby an increased translation and, thus, accelerated turnover of RNA might be promoted by
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13 400 ingesting a combination of carbohydrates and proteins following exercise. An improved anabolic-catabolic
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15 401 balance favored by ingestion of carbohydrates and proteins is supported by the marked decrease of 3-
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17 402 methylhistidine (3-MeHis) observed following ingestion of LCHO-P as compared to ingestion of water or
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19 403 carbohydrate containing beverages. 3-MeHis is a methylated amino acid derived mainly from actin and myosin,
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21 404 and following catabolism, the only fate of 3-MeHis is excretion in urine⁵⁵. Approximately 90% of the total
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23 405 human 3-MeHis has been estimated to be derived from skeletal muscle⁵⁶. Thus, 3-MeHis in urine is a known
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25 406 marker of the degradation rate of myofibrillar protein, an intake of carbohydrates in combination with
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27 407 essential amino acids attenuates the increase in 3-MeHis following exercise⁵⁷. Thus, the reduced level of 3-
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29 408 MeHis observed points to a lower whole body proteolysis including myofibrillar catabolism as an effect of
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31 409 combined ingestion of carbohydrates and proteins.
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35 410 Another interesting finding from the metabolomic data were the difference in metabolic response in the
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37 411 recovery phase from exercise, following water ingestion, when the five subjects with highest (mean 68 mL • kg⁻¹ • min⁻¹;
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39 412 HighFit) and lowest (mean 49 mL • kg⁻¹ • min⁻¹; LowFit) VO₂peak respectively were subject to
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41 413 multivariate comparison (Figure 5). Here, a greater anabolic drive was indicated by an increased level of PSU in
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43 414 the HighFit group. Additionally, a higher serum concentration of uric acid (UA) was also seen in this group.
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45 415 Elevated level of UA in the early recovery phase from exercise is a major consequence of adenine catabolism in
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47 416 muscle induced during exercise⁵⁸. Thus the increased UA concentration seen in the HighFit group is likely to be
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49 417 related to adenine catabolism, due to the higher total work-load subjected on these individuals during the
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51 418 ergometer cycling. Alternatively, a greater uptake of uric acid from blood to stressed tissues can explain the
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53 419 lower serum uric acid level seen in the LowFit group. During exercise there is an increase in production of
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55 420 reactive oxygen species (ROS) in tissues stressed by exercise and systemic uric acid is utilized as an antioxidant
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57 421 in skeletal muscle⁵⁹.
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3 422 Interestingly, when the five LowFit subjects ingested LCHO-P their systemic metabolic profile was
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5 423 changed towards the profile of the HighFit group following ingestion of water. This change in metabolic pattern
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7 424 can likely be explained by reduced levels of catabolic markers such as fatty acids and increased level of sugars,
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9 425 amino acids, insulin and PSU, indicating less catabolism and hence a favored anabolic state. One LowFit subject
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11 426 revealed a deviating response at two time points (i.e. 15 and 30 min post exercise) (Figure 5A) when ingesting
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13 427 LCHO-P. This was one of two subjects, the other subject was not included in the LowFit vs HighFit modelling,
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15 428 which diverged in insulin response with the ingestion of carbohydrates and carbohydrates + proteins
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17 429 suggesting that this could potentially be an early indication of impaired insulin function or insulin resistance.
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19 430 The observed parallel increase in myo-inositol level in this subject (and in the other subject showing an
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21 431 abnormal insulin response) could be a consequence of increased availability of glucose following carbohydrate
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23 432 intake, which may promote production of myo-inositol. This finding could potentially support the hypothesis of
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25 433 an early indication of impaired insulin function or insulin resistance. Although being an extremely interesting
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27 434 result captured from the metabolomic data, it still remains to be investigated and verified in additional subjects
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29 435 and investigated in detail on in relation to metabolic changes before drawing too bold conclusions.
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33 436 **Predictive metabolomics approach**

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35 437 The usefulness of the predictive metabolomics approach was clearly demonstrated as an informative
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37 438 complement to the analysis of single metabolites or combined measurements of specific compound classes,
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39 439 e.g. FFA combined profiles. Apart from the fact that metabolomics can work as a hypothesis verifying as well as
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41 440 a hypothesis generating tool, it was shown that the metabolomics data provided a huge amount of additional
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43 441 systematic variation compared to the measured clinical parameters (insulin, FFA and glucose). Furthermore,
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45 442 the use of chemometric methods provided multivariate maps of subject and metabolite patterns which made it
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47 443 possible to overview and investigate the complex metabolic interactions occurring in blood serum following
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49 444 exercise and nutritional modulation. This is of value for generating mechanistic explanations based on the
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51 445 whole set of small molecules detected in a system. An important point is that in the search for specific
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53 446 biomarkers in various sets of omics data chemometric methods are not superior to other statistical methods.
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55 447 However for detecting biomarker patterns or providing interpretations of multivariate interactions they are a
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57 448 prerequisite. It should be pointed out that we haven't made a full interpretation of all the changes in the data,
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59 449 but rather focused on the most interesting findings. One reason for this is that not all detected metabolites
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3 450 could be given an identity, which highlights one drawback with the metabolomics technology at present. The
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5 451 issue of identifying a larger proportion of the detected metabolites is one of great importance for the future
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7 452 and also one of the most intriguing challenges, since this is likely to reveal many new interesting findings in
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9 453 various important applications.

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12 454 A crucial issue for life science applications and especially where omics analyses have been applied is the
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14 455 validation of models as well as verification of detected biomarkers/biomarker patterns and mechanistic
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16 456 findings over multiple studies. If this is done thoroughly the chances of generating useful results and methods
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18 457 will increase. The predictive metabolomics approach applied here provides a framework for validation of both
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20 458 the data processing (HMCR) and the multivariate models. This predictive feature of the methodology made it
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22 459 possible to validate the models and the detected patterns using independent samples in a reliable and
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24 460 transparent way. In addition, cross validation was used as an internal validation for all models meaning that all
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26 461 the presented results are based on predicted values, which dramatically decreases the risk of overfitting and
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28 462 increases the reliability of the findings. A major and unique benefit of the predictive metabolomics approach is
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30 463 the possibility to perform verifications of detected metabolite patterns over multiple studies, which is crucial
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32 464 for establishing novel hypothesis or verifying the diagnostic value of a detected metabolite pattern. In our
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34 465 belief, this is an accurate and efficient approach to obtain interpretable and validated results, based on high
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36 466 quality metabolomics data. This approach could be of value when moving towards future goals, set by the
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38 467 omics community, such as diagnostic or prognostic tools, systems biology modelling or even personalized
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40 468 medicine and nutrition. The benefits of the methodology was further emphasised in the classification of
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42 469 subjects in relation to fitness level followed by nutritional modulation by LCHO-P. In this example it was clearly
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44 470 shown how individual modulations could be monitored based on concentration changes in hundreds of low
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46 471 molecular weight compounds. Of great importance for obtaining a reliable interpretation of individual
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48 472 responses in humans is the use of each included subject as its own control. In this study we utilized the sample
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50 473 taken directly after finishing exercise as the individual reference and normalized all the other time points to
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52 474 this sample. In this way we could get a detailed investigation of each individual's metabolic response over time
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54 475 as well as a reliable comparison of this response between subjects. This highlights how the approach could be
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56 476 used for selecting, monitoring and controlling individual treatments, but also to detect individuals showing a
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58 477 different response to various treatments, e.g. slow and fast responders.
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Conclusion

The predictive metabolomics approach based on a combination of predictive HMCR processing of acquired GC/TOFMS data and chemometric data analysis by means of OPLS was proven valuable for studying and validating the complex interactions occurring in human serum induced by nutritional modulation following physical exercise. Pseudouridine (PSU) was suggested as a novel marker for pro-anabolic effect following ingestion of LCHO-P. This was explained by a combination of elevated insulin concentration and an increased availability of amino acids for protein synthesis, leading to an increased translation and, thus, accelerated turnover of RNA. The result was further verified by the marked decrease of 3-methylhistidine (3-MeHis) observed in the same samples indicating a lower whole body proteolysis including myofibrillar catabolism as an effect of combined ingestion of carbohydrates and proteins. Metabolic differences in relation to fitness level was also detected. This was partly explained by a greater anabolic drive, indicated by an increased level of PSU, as well as an increased adenine catabolism, indicated by elevated levels of uric acid (UA) in the HighFit group. Alternatively, a greater uptake of UA from blood to stressed tissues could explain the lower UA levels in the LowFit group. Multivariate monitoring of nutritional modulation revealed that LowFit subjects ingesting LCHO-P moved in metabolic profile towards the HighFit group subjects having ingested water. This change in metabolic pattern was explained by reduced levels of catabolic markers such as fatty acids and increased level of sugars, amino acids, insulin and PSU, indicating less catabolism and hence a favored anabolic state. Finally, changes in the metabolomic data indicative of possible early signs of impaired insulin function or insulin resistance were detected following ingestion of carbohydrates or carbohydrates + proteins. However, this extremely interesting finding remains to be verified in additional subjects in a dedicated study.

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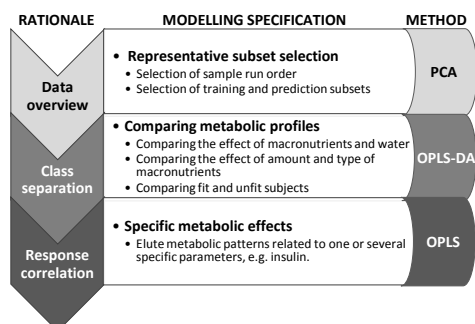
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689 **Figures and tables**

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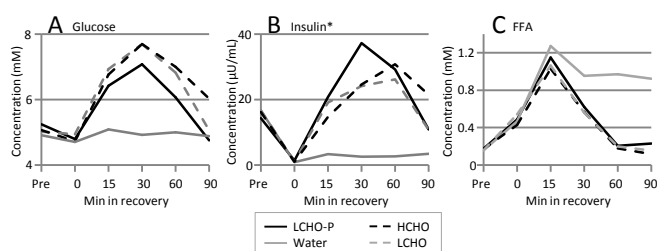


692

693 **Figure 1.** An overview of the applied multivariate statistic methods, i.e. principal component analysis (PCA), orthogonal partial least

694 squares (OPLS) and OPLS-discriminant analysis (OPLS-DA).

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696

697 **Figure 2.** The arithmetic mean concentration of glucose (left), insulin (center) and free fatty acids (FFA) (right). Black line corresponds

698 to subjects ingesting low carbohydrate-protein (LCHO-P) beverage immediately after performed exercise, grey line corresponds to subjects

699 ingesting water, dashed black line subjects ingesting high carbohydrates (HCHO) and dashed grey line to subjects ingesting low

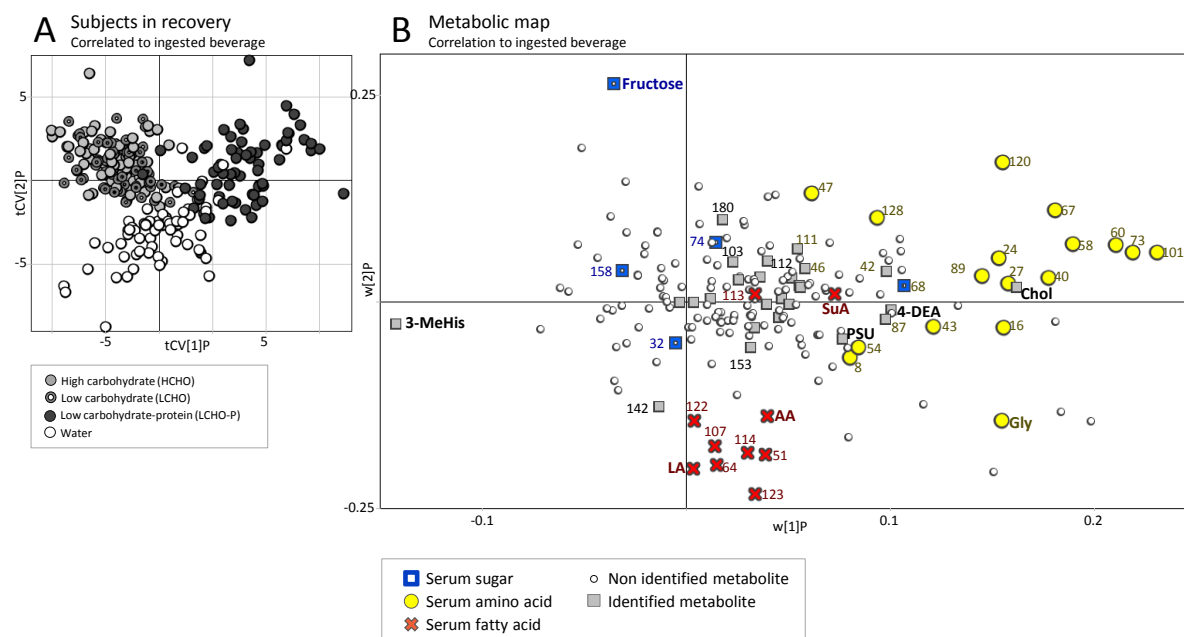
700 carbohydrate beverage (LCHO). *Two subjects were excluded when calculating the mean insulin concentration due to a deviating insulin

701 response when ingesting carbohydrate containing beverages after completed exercise.

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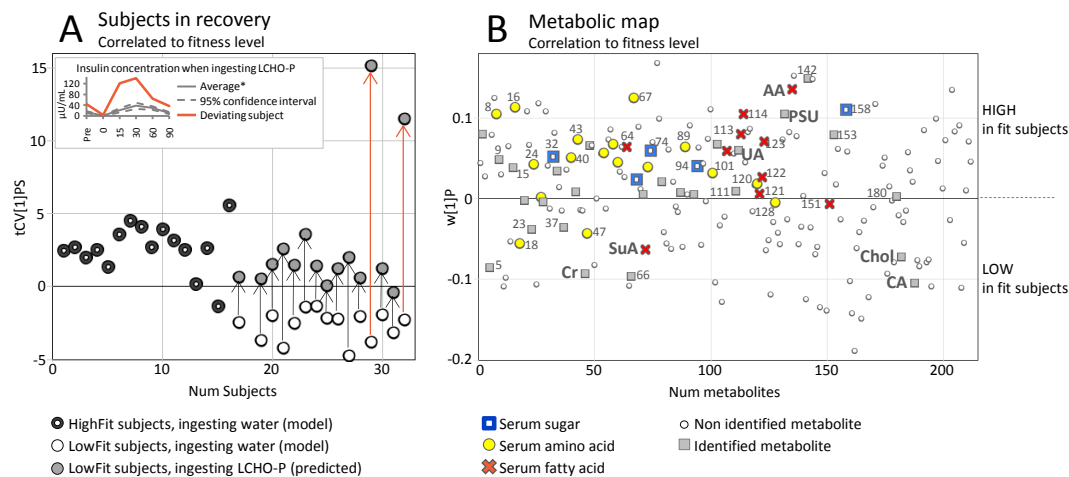
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706 **Figure 3. (A)** Cross-validated scores (tCV) from an OPLS-DA model describing the first two predictive components revealing clustering
 707 of subjects in relation to their ingested beverage following exercise. The model includes all recovery samples taken after nutritional intake,
 708 i.e. 15, 30, 60 and 90min after completed exercise. Each item in the plot corresponds to a subject at a specific time point post ingestion of
 709 either a high carbohydrate (HCHO), low carbohydrate (LCHO), low carbohydrate-protein (LCHO-P) or water after finished exercise. **(B)** An
 710 OPLS-DA covariance loading plot of the resulting metabolic patterns explaining the pattern seen in the above mentioned score plot ($w[1]P$
 711 and $w[2]P$ are covariance loadings for the first and second predictive component respectively). Each item corresponds to a potential
 712 metabolite coloured according to its class belonging, see figure legend. More information regarding all identified metabolites is presented
 713 in **table 1**.

714

715 **Figure 4.** The relative mean concentration of resolved 3-methylhistidine (3-MeHis) **(A)** and pseudouridine (PSU) **(B)**, from the GC-
 716 TOF/MS data, after normalisation by means of subtracting the detected concentration immediately after performed exercise (0min) from
 717 the corresponding recovery time points, i.e. 15, 30, 60 and 90min in recovery. 3-MeHis decreases when ingesting a low carbohydrate-
 718 protein beverage while PSU increases, compared to the sole ingestion of carbohydrate or water.

719



720

721 **Figure 5. (A)** Cross validated scores from a calculated OPLS-DA model describing the separation of subjects with high and low fitness

722 level in the early recovery phase when ingesting water, i.e. samples taken 15, 30, 60 and 90min after performed exercise. Subjects with

723 high fitness level (HighFit) are plotted as black circles while subjects with low fitness level (LowFit) are plotted as white dots. Corresponding

724 LowFit subjects ingesting low carbohydrate-protein beverage (LCHO-P) following exercise were predicted into existing model and plotted

725 here as grey dots. The corresponding LowFit subjects' are connected with arrows. Inserted figure: The Insulin concentration of LowFit

726 subject with a deviating metabolic pattern when ingesting LCHO-P. **(B)** The regression coefficient plot, i.e. a metabolic map explaining the727 differences related to subjects fitness level ($w[1]P$ is the covariance loadings for the predictive component). Information regarding728 identified metabolites is presented in **table 1**.

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Table 1. Identified metabolites. ^aMetabolites were analyzed as methyloxime-trimethylsilyl derivatives (MeOx TMS). ^bMass spectra match values according to NIST MS-Search 2.0. ^cMass spectra search were done with in-house libraries and the Max Planck Institute (MPI) library in Golm (<http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html>). ^d“–” is that retention time index (RI) information in the library is not available.

ID	Derivatized metabolites ^a	Abbreviation	Match value ^{b,c}	RI-RI _{library} ^{c,d}
5	Heptanoic acid (TMS)	HepA	851	2
8	Valine (2TMS)	Val	897	1
9	Ethanolamine (3TMS)	ETA	768	-
15	Phosphoric acid (3TMS)	PhA	863	1
16	Isoleucine (2TMS)	Ile	873	1
18	Glycine (3TMS)	Gly	937	3
20	Glyceric acid (3TMS)	GlyA	774	2
23	4-Deoxyerythronic acid (3TMS)	4-DEA	712	-
24	Serine (3TMS)	Ser	916	1
27	Threonine (3TMS)	Thr	957	5
32	Erythrose methoxyamine (3 TMS)	Erythrose	647	4
37	Malic acid (3TMS)	MalA	748	2
40	Methionine (2TMS)	Met	755	2
42	Pyroglutamic acid (2TMS)	PGA	922	7
43	Hydroxyproline (3TMS)	HyP	807	1
46	Creatinine (3TMS)	Cr	899	2
47	Cysteine (3TMS)	Cys	914	1
48	Threonic acid (4TMS)	THRA	811	14
54	Glutamine (4TMS) + (3TMS)	Glu	869 / 958	3
58	Ornithine (3TMS) + (4TMS)	Orn	764 / 964	2
60	Phenylalanine (2TMS)	Phe	939	1
64	Lauric acid (TMS)	LauA	848	1
66	Taurine (3TMS)	Tau	928	4
67	Asparagine (3TMS)	Asp	958	0
68	Ribose (MeOx4TMS)	Ribose	726	21
72	Suberic acid (TMS)	SUA	800	17
73	Lysine (3TMS) + (4TMS)	Lys	673 / 898	4
74	Arabitol (5TMS)	Arabitol	887	4
79	Glycerol-3-phosphate (4TMS)	Gly-3-P	926	2
87	Citric acid (4TMS)	CA	956	4
89	Arginine (5TMS)	Arg	843	15
93	3-Methylhistidine (2TMS)	3-MeHis	616	0
94	Fructose (MeOx 5TMS)	Fructose	969	3
101	Tyrosine (3TMS)	Tyr	931	1
103	Indole-3-acetic acid (2TMS)	IAA	698	0
107	9-(Z)-Hexadecenoic acid (TMS)	9-(Z)-HexA	920	--
111	myo-Inositol (6TMS)	MI	976	7
112	Uric acid (4TMS)	UA	958	3

114	Palmitic acid (TMS)	PA	779	-
120	Tryptophan (3TMS)	Trp	754 / 697	1
121	Linoleic acid (TMS)	LA	943	2
122	Oleic acid (TMS)	OA	961	10
123	Stearic acid (TMS)	SA	967	7
128	Cystine (4TMS)	Cystine	954	1
132	Pseudouridine (5TMS)	PSU	808	-
135	Arachidonic acid (TMS)	AA	901	4
151	Docosahexanoic acid (TMS)	DHA	925	4
153	Inosine (4TMS)	Inosine	869	33
158	Sucrose (8TMS)	Sucrose	842	5
180	Adenosine-5-monophosphate (5TMS)	5-AMP	877	26
182	Cholesterol (TMS)	Chol	913	4
188	Cholic acid (4TMS)	CA	872	3

734

735 **Table 2.** Results from calculated regression models. All models are OPLS-DA models if not stated otherwise. Model samples from subjects
 736 ingesting a low carbohydrate beverage samples are denoted LCHO, high carbohydrate beverage HCHO, low carbohydrate-protein beverage
 737 LCHO-P and water W. The R2X value is the explained variation in model samples (X) and R2X_{ycorr} is the amount of variation in X which is
 738 correlated to Y (response matrix, i.e. a dummy matrix in OPLS-DA modelling). The Q2 value describes the predictive ability of the model,
 739 based on sevenfold-cross validation.

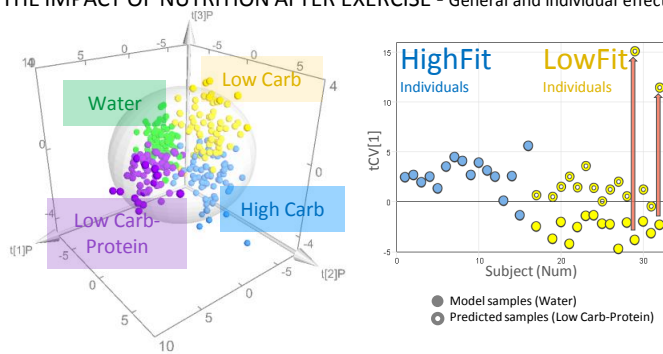
Time point specific models (beverage1:beverage2 at min in recovery)	No comp	R2X (%)	R2X _{ycorr} (%)	R2Y (%)	Q2 (%)	Specificity (% correct predictions)	Selectivity (% correct model samples)	Num model samples	Num predicted samples
LCHO-P:W +15	1P+10	27.7	8.52	79.4	27.1	64	97	36 (18LCHO-P+18W)	11
LCHO-P:W +30	1P+10	0.226	8.22	84.9	35.8	100	100	34 (17LCHO-P+17W)	11
LCHO-P:W +60	1P+10	0.342	11.6	70	44.1	75	97	34 (17LCHO-P+17W)	12
LCHO-P:W +90	1P+20	36.6	9.49	92.3	66.6	73	100	34 (17LCHO-P+17W)	11
HCHO:W +15	1P	6.5	6.5	74.2	30.6	50	94	34 (18HCHO+16W)	8
HCHO:W +15	1P+20	27.6	4.28	93.7	40.1	75	100	34 (18HCHO+16W)	8
HCHO:W +30	1P+20	34.7	6.31	92.2	64.8	90	100	35 (18HCHO+17W)	10
HCHO:W +60	1P+20	45.1	6	94.2	73.6	64	100	35 (18HCHO+17W)	11
HCHO:W +90	1P+20	34.4	7.15	97.1	84	100	100	35 (18HCHO+17W)	11
LCHO:W +15	-	-	-	-	-	-	-	-	-
LCHO:W +30	1P+20	38.8	63.3	93	69.1	90	100	35 (18LCHO+17W)	10
LCHO:W +60	1P+20	41.9	5.39	94.4	68	70	100	35 (18LCHO+17W)	10
LCHO:W +90	1P+20	36.3	5.58	95.5	69.7	90	100	35 (18LCHO+17W)	10
LCHO-P:LCHO +15	1P+10	30.6	8.92	84	54.7	78	97	35 (17LCHO-P+18LCHO)	9
LCHO-P:LCHO +30	1P+10	34	11.1	90.3	67.6	100	100	35 (17LCHO-P+18LCHO)	11
LCHO-P:LCHO +60	1P+10	35.8	7.84	85.6	64.3	50	100	35 (17LCHO-P+18LCHO)	10
LCHO-P:LCHO +90	1P+10	31.3	6.99	81.1	50	60	100	35 (17LCHO-P+18LCHO)	10
LCHO-P:HCHO +15	1P+10	25.9	9.81	89.1	58.9	78	100	35 (18HCHO+17LCHO-P)	9
LCHO-P:HCHO +30	1P+20	34.7	9.21	97.4	73.9	100	100	35 (18HCHO+17LCHO-P)	11
LCHO-P:HCHO +60	1P+20	43.2	7.45	95.9	78.9	73	100	35 (18HCHO+17LCHO-P)	11
LCHO-P:HCHO +90	1P+10	25.9	8.4	83.1	59.5	82	100	35 (18HCHO+17LCHO-P)	11

Recovery models	Num comp	R2X (%)	R2Xycorr (%)	R2Y (%)	Q2 (%)	Specificity (% correct predictions)	Selectivity (% correct model samples)	Num model samples	Num predicted samples
HighFit-LowFit	1P+10	22.8	7.67	90.5	74.1	-	100	37 (16HighFitF-21LowFit)	-
OPLS model (Y=insulin, FFA and Cortisol)	3P+30	40.4	9.48	65.4	49.7	-	-	271 (67LCHO-P+67W+71HCHO+66LCHO)	-
All beverages	3P+50	45.3	9.06	66.2	52.1	-	-	279 (68LCHO-P+67W+72HCHO+72LCHO)	-
All beverages (excluded amino acids)	3P+40	40.8	8	61.7	47.9	-	-	279 (68LCHO-P+67W+72HCHO+72LCHO)	-

740

741 Synopsis

THE IMPACT OF NUTRITION AFTER EXERCISE - General and individual effects



742

743 By using the predictive metabolomics approach, we show that the composition of macronutrients ingested
 744 immediately after exercise affects the serum metabolic profile in the early recovery phase. More specific, pro-
 745 anabolic processes are favored when ingesting a carbohydrate-protein mix compared to the sole intake of
 746 water or carbohydrates. Additional findings are presented related to the detection of early insulin resistance
 747 and the possibility for individual nutrient modulation.