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## Predicting physiological imbalance in Holstein dairy cows by three different sets of milk biomarkers

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### Abstract

Blood biomarkers may be used to detect physiological imbalance and potential disease. However, blood sampling is difficult and expensive, and not applicable in commercial settings. Instead, individual milk samples are readily available at low cost, can be sampled easily and analysed instantly. The present study sampled blood and milk from 234 Holstein dairy cows from six experimental herds in different European countries. The objective was to compare the use of three different sets of milk biomarkers for identification of cows in physiological imbalance and thus at risk of developing a metabolic or infectious disease. Random forests was used to predict body energy balance (EBAL), index for physiological imbalance (PI-index) and three clusters differentiating the metabolic status of cows created on basis of concentrations of plasma glucose, plasma  $\beta$ -hydroxybutyrate (BHB), plasma non-esterified fatty acids (NEFA) and serum IGF-1. These three metabolic clusters were interpreted as cows in balance, cows in physiological imbalance and "intermediate cows" with a physiological status in between. The three sets of milk biomarkers used for prediction were: milk Fourier transform mid-IR (FT-MIR) spectra, 19 immunoglobulin G (IgG) N-glycans and 8 milk metabolites and enzymes (MME). Blood biomarkers were sampled twice; around 14 days after calving (days in milk (DIM)) and around 35 DIM. MME and FT-MIR were sampled twice weekly 1-50 DIM whereas IgG N-glycan were measured only four times. Performances of random forests predictions for EBAL and PI-index were measured by the coefficient of determination ( $R^2_{cv}$ ) and the root mean squared error (RMSE<sub>cv</sub>) from leave-one-cow-out (internal) cross-validation (CV). For metabolic clusters, performance was measured by sensitivity, specificity and global accuracy from this cross-validation. Neither EBAL nor PI-index were sufficiently precise to be used as a management tool for identification of risk cows. The best prediction of PI-index was obtained by MME ( $R^2_{CV} = 0.40$  at 14 DIM and  $0.35$  at 35 DIM) while FT-MIR showed a better performance than MME for prediction of EBAL ( $R^2_{CV} = 0.28$  vs  $0.21$ ). Global accuracies of predicting metabolic clusters from MME and FT-MIR were at the same level and ranged from  $0.54$  to  $0.65$  for MME and  $0.51$  to  $0.68$  for FT-MIR.  $R^2_{CV}$  and accuracies were lower for IgG N-glycans. In conclusion, MME and FT-MIR can be used to predict the physiological status of the cows, while the use of IgG N-glycans for prediction still needs development.

<b>Keywords</b>	Metabolites; enzymes; FT-MIR; IgG N-glycans; metabolic clusters; random forests
<b>Taxonomy</b>	Animal Lactation, Dairy Cattle, Animal Energetics, Animal Metabolism
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To the Editor

Preventive Veterinary Medicine

Currently, the prediction at a large scale of physiological status of cows is of great interest in order to perform genetic studies and for the management of cows. The use of milk biomarkers seems a good strategy as it is easily accessible and already routinely collected. Enclosed please find our manuscript entitled "Predicting physiological imbalance in Holstein dairy cows by three different sets of milk biomarkers" authored by Foldager et al. This manuscript was developed in the frame of the GplusE project granted by the European Union, which sampled blood and milk from 234 Holstein dairy cows from six experimental herds in different European countries. The objective was to compare the use of three different sets of milk biomarkers for identification of cows in physiological imbalance and thus at risk of developing a metabolic or infectious disease. Milk biomarkers used are metabolites and enzymes, Fourier transform mid-infrared (FT-MIR) spectra and immunoglobulin G (IgG) *N*-glycans. Based on the same data, two other papers from the GplusE project (De Koster et al., 2019; Grelet et al., 2019) have considered the prediction of metabolic status (balanced/unbalanced) using metabolic clusters based on k-means clustering of four blood biomarkers; glucose, non-esterified fatty acids (NEFA) and  $\beta$ -hydroxybutyrate (BHB) in plasma and insulin-like growth factor-1 (IGF-1) in serum. A third paper from the GplusE project (Krogh et al., accepted 26 Sep 2019) focused on herd variation in the biomarkers. The present paper brings new knowledge by comparing random forest predictions of body energy balance (EBAL), index for physiological imbalance (PI-index) and the metabolic clusters just described. The paper goes deeper in the evaluation of the potential of milk metabolites and enzymes but also investigate the potential of IgG *N*-glycans as biomarker and contributes to the understanding of the clustering approach. The main objective was to compare the use of milk metabolites and enzymes, FT-MIR spectra and IgG *N*-glycans for identification of cows in physiological imbalance and thus at risk of developing a metabolic or infectious disease.

We hope you will consider this paper for publication in Preventive Veterinary Medicine.

Yours sincerely,

Leslie Foldager, PhD, MSc  
Senior Researcher  
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Aarhus University, Tjele, Denmark

## 1 **Highlights**

- 2 • Identifying physiological imbalance/disease risk in dairy cows for herd  
3 management
- 4 • Blood biomarkers are relevant indicators but not generally applicable  
5 commercially
- 6 • Milk biomarkers can be taken automatically as in Herd Navigator™
- 7 • FT-MIR spectra and milk metabolites and enzymes appeared equally good as  
8 biomarkers
- 9 • IgG *N*-glycans suffered from fewer samples and completeness and needs  
10 development

1 **Predicting physiological imbalance in Holstein dairy cows by three different**  
2 **sets of milk biomarkers**

3

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29

30

31 **Abstract**

32 Blood biomarkers may be used to detect physiological imbalance and potential  
33 disease. However, blood sampling is difficult and expensive, and not applicable in  
34 commercial settings. Instead, individual milk samples are readily available at low  
35 cost, can be sampled easily and analysed instantly. The present study sampled  
36 blood and milk from 234 Holstein dairy cows from six experimental herds in different  
37 European countries. The objective was to compare the use of three different sets of  
38 milk biomarkers for identification of cows in physiological imbalance and thus at risk  
39 of developing a metabolic or infectious disease. Random forests was used to predict  
40 body energy balance (EBAL), index for physiological imbalance (PI-index) and three  
41 clusters differentiating the metabolic status of cows created on basis of  
42 concentrations of plasma glucose, plasma  $\beta$ -hydroxybutyrate (BHB), plasma non-  
43 esterified fatty acids (NEFA) and serum IGF-1. These three metabolic clusters were  
44 interpreted as cows in balance, cows in physiological imbalance and “intermediate  
45 cows” with a physiological status in between. The three sets of milk biomarkers used  
46 for prediction were: milk Fourier transform mid-IR (FT-MIR) spectra, 19  
47 immunoglobulin G (IgG) *N*-glycans and 8 milk metabolites and enzymes (MME).  
48 Blood biomarkers were sampled twice; around 14 days after calving (days in milk  
49 (DIM)) and around 35 DIM. MME and FT-MIR were sampled twice weekly 1-50 DIM  
50 whereas IgG *N*-glycan were measured only four times. Performances of random

51 forests predictions for EBAL and PI-index were measured by the coefficient of  
52 determination ( $R^2_{cv}$ ) and the root mean squared error ( $RMSE_{cv}$ ) from leave-one-cow-  
53 out (internal) cross-validation (CV). For metabolic clusters, performance was  
54 measured by sensitivity, specificity and global accuracy from this cross-validation.  
55 Neither EBAL nor PI-index were sufficiently precise to be used as a management tool  
56 for identification of risk cows. The best prediction of PI-index was obtained by MME  
57 ( $R^2_{CV} = 0.40$  at 14 DIM and 0.35 at 35 DIM) while FT-MIR showed a better  
58 performance than MME for prediction of EBAL ( $R^2_{CV} = 0.28$  vs 0.21). Global  
59 accuracies of predicting metabolic clusters from MME and FT-MIR were at the same  
60 level and ranged from 0.54 to 0.65 for MME and 0.51 to 0.68 for FT-MIR.  $R^2_{CV}$  and  
61 accuracies were lower for IgG *N*-glycans. In conclusion, MME and FT-MIR can be  
62 used to predict the physiological status of the cows, while the use of IgG *N*-glycans  
63 for prediction still needs development.

64

### 65 **Abbreviations**

66 BHB,  $\beta$ -hydroxybutyrate; CV, cross-validation; DIM, days in milk; EBAL, body energy  
67 balance; FT-MIR, Fourier transform mid-IR; IgG, immunoglobulin G; LDH,  
68 dehydrogenase; MME, metabolites and enzymes; NAGase, *N*-acetyl- $\beta$ -D-  
69 glucosaminidase; NEFA, non-esterified fatty acids; PI-index, index for physiological  
70 imbalance;  $R^2$ , coefficient of determination; RMSE, root mean squared error; VIM,  
71 variable importance measures

72

### 73 **Keywords**

74 Metabolites; enzymes; FT-MIR; IgG *N*-glycans; metabolic clusters; random forests

75

76 **Introduction**

77 Diseases at calving and during early lactation account for the majority of health and  
78 welfare problems in dairy production (Ingvarsten et al., 2003). These include  
79 production diseases such as fatty liver, ketosis, rumen acidosis and lameness. Most  
80 of such diseases in periparturient cows are argued to be the result of physiological  
81 imbalance (Ingvarsten, 2006). Correspondingly, infectious diseases such as mastitis  
82 and metritis are included as the immune system is strongly interlinked with  
83 physiological imbalance via the endocrine system and metabolites that must  
84 accommodate to the demands for lactation facing the transition cow (Ingvarsten and  
85 Moyes, 2015). The consequences of subclinical and clinical diseases are suboptimal  
86 animal welfare and production and lower reproductive efficiency. Thus, physiological  
87 imbalance leading to these subclinical and clinical diseases should have high priority  
88 of being addressed with regard to development of management tools.

89  
90 Cows in physiological imbalance have increased risk of developing diseases and  
91 reduced production (Ingvarsten et al., 2003; Bjerre-Harpoth et al., 2012). Subclinical  
92 stages of diseases can be detected by biomarkers while the cow may appear  
93 completely healthy. A number of biomarkers in blood are well described but are  
94 currently less well characterized in milk. In the review of Ingvarsten (2006), it is  
95 documented that plasma concentrations of glucose, non-esterified fatty acids (NEFA)  
96 and  $\beta$ -hydroxybutyrate (BHB) are relevant indicators to determine subclinical ketosis.  
97 LeBlanc et al. (2005) also identified blood NEFA and BHB as relevant indicators of  
98 displaced abomasum in dairy cows. Piechotta et al. (2012) reported that  
99 concentrations of serum NEFA and plasma IGF-1 prepartum are associated with  
100 postpartum diseases, while IGF-1 postpartum was the best predictor of both left

101 displaced abomasum and risk of culling (Lyons et al., 2014). However, collecting and  
102 analysing blood samples for measuring biomarkers is difficult and expensive, and not  
103 applicable in commercial settings. Instead, individual milk samples are readily  
104 available and milking systems even provide automatic sampling and measurement of  
105 e.g. milk conductivity. Such automatic systems can be expanded to measure e.g.  
106 milk BHB (e.g. Herd Navigator™, <http://www.herdnavigator.com>).

107

108 Enjalbert et al. (2001) showed that subclinical ketosis can be identified by measuring  
109 BHB in milk with enzymatic analysis or with Ketolac test strips. Other studies also  
110 reported milk BHB to be a relevant indicator of subclinical and clinical ketosis (e.g.  
111 Nielsen et al., 2005). Free glucose, glucose-6-phosphate (Larsen and Moyes, 2015),  
112 and isocitrate (Larsen, 2014) reflect the nutrient availability and metabolic turnover in  
113 the mammary gland that are linked to the blood levels and therefore potentially  
114 indicators of physiological imbalance and risk of disease. Larsen et al. (2010) and  
115 Kitchen et al. (1978), respectively, reported that the milk enzymes lactate  
116 dehydrogenase (LDH) and *N*-acetyl- $\beta$ -D-glucosaminidase (NAGase) performed  
117 equally with somatic cell count and acute phase proteins as inflammatory indicators  
118 of mastitis. In addition, Fourier transform mid-IR (FT-MIR) spectra of milk can be  
119 calibrated to estimate e.g. milk metabolites, and measures of milk immunoglobulin G  
120 (IgG) *N*-glycans may be potential new biomarkers.

121

122 Based on the same data as here, two other papers (De Koster et al., 2019; Grelet et  
123 al., 2019) have considered the prediction of metabolic status (balanced/unbalanced)  
124 using metabolic clusters based on k-means clustering of four blood biomarkers;  
125 glucose, NEFA and BHB in plasma and IGF-1 in serum. The present paper

126 supplements these papers by comparing random forests predictions from three  
127 different sets of milk biomarkers; metabolites and enzymes (MME), FT-MIR spectra  
128 and IgG *N*-glycans. In addition to metabolic clusters, predictions of body energy  
129 balance (EBAL) and index for physiological imbalance (PI-index) (Ingvarsten, 2006;  
130 Moyes et al., 2013a, 2013b) were considered. Grelet et al. (2019) used a different  
131 prediction method and only considered FT-MIR, De Koster et al. (2019) only used  
132 multiparous cows and both studies only considered prediction of clusters.

133

134 The present paper focuses more on MME but also investigate the potential of IgG *N*-  
135 glycans as a set of milk biomarkers and contributes to the understanding of the  
136 clustering approach. The main objective was to compare the use of MME, FT-MIR  
137 and IgG *N*-glycans for identification of cows in physiological imbalance and thus at  
138 risk of developing a metabolic or infectious disease.

139

## 140 **Material and methods**

141 Study design, sampling and analysis of milk as well as blood have been described in  
142 De Koster et al. (2019), Grelet et al. (2019) and Krogh et al. (2019). In brief, six  
143 experiments were conducted in Northern Ireland (UK), Denmark (DK), Belgium (BE),  
144 Italy (IT), Germany (DE) and Ireland (IE). These included a total of 234 Holstein dairy  
145 cows (55 first parity, 66 second parity, and 113 in third or higher parity (3+), see  
146 Supplementary Table S1). In four experiments, all cows were fed a standard diet  
147 typical for the particular country. In the UK and DK experiments, a standard diet and  
148 two different experimental diets were used. An overview of the diets is shown in table  
149 1 of Krogh et al. (2019).

150

151 *Derived measures*

152 The calculation of EBAL was described in De Koster et al. (2019) and Krogh et al.  
153 (2019). EBAL was only calculated if both morning and evening yield was available for  
154 that day. Afterwards, three days (i.e. +/- 1 days in milk (DIM)) moving averages of  
155 EBAL were calculated and used for the analyses. The average live body weights  
156 within calendar week was used to smooth large day-to-day variation and  
157 measurement errors of scales. Summary statistics of EBAL are shown in  
158 supplementary tables of Krogh et al. (2019).

159

160 PI-index was calculated as  $[\log_{10}(\text{NEFA})] + [\log_{10}(\text{BHB})] - [\text{glucose}]$  (Moyes et al.,  
161 2013a), where plasma concentrations of the individual metabolites were standardised  
162 to an overall mean of zero and variance of one (as indicated by square brackets).  
163 Moyes et al. (2013a) used the natural logarithm ( $\ln$ ) but since  $\log_{10}$  and  $\ln$  are  
164 proportional,  $\ln(y) = \ln(10)\log_{10}(y)$ , the standardised values will be exactly equal, i.e.  
165  $[\ln(y)] = [\log_{10}(y)]$ . Thus, since the manuscripts of Grelet et al. (2019) and De Koster  
166 et al. (2019) applied  $\log_{10}$ -transformations of NEFA and BHB we decided to continue  
167 this approach.

168

169 *Metabolic clusters*

170 As an alternative phenotype to negative EBAL and PI-index, clusters were created by  
171 use of the k-means method of Hartigan and Wong (1979) from standardised  
172 measures of plasma glucose, plasma  $\log_{10}(\text{BHB})$ , plasma  $\log_{10}(\text{NEFA})$ , and serum  
173  $\log_{10}(\text{IGF-1})$ . As mentioned in the Introduction, these four blood biomarkers mirror the  
174 physiological status of the animal. Three clusters ( $k=3$ ) were constructed for each  
175 combination of three parities (1, 2 and 3+ lactations) and two periods in early

176 lactation (around 14 and 35 DIM) as visualised in Figure 1. Deciding on the number  
177 of clusters can be intricate but in the present sample  $k=3$  was found to be a fair  
178 compromise between size and similarity (in terms of the within cluster sum of  
179 squares, results not shown). Based on a graphical interpretation using boxplots of the  
180 standardised concentrations of plasma glucose, NEFA and BHB and serum IGF-1  
181 (see Figure 1) three metabolic clusters were defined as representing balanced,  
182 intermediate and imbalanced cows.

183  
184 Criteria to define the imbalanced metabolic cluster are the most important. We  
185 defined the metabolic cluster as imbalanced if standardised plasma glucose and  
186 serum IGF-1 concentrations were both lower than those of plasma BHB and plasma  
187 NEFA, and in addition both median BHB and NEFA were above 0.5 SD (Figure 1).  
188 Intermediate and balanced metabolic clusters had less sharp definitions: The  
189 intermediate metabolic cluster generally had lower standardised glucose and IGF-1  
190 concentrations than BHB and NEFA, with NEFA and BHB boxes in the  $\pm 0.5$  SD area  
191 and glucose and IGF-1 around or below  $-0.5$  SD. The balanced metabolic cluster had  
192 standardised glucose and IGF-1 concentrations around 0.5 SD and standardised  
193 NEFA and BHB concentrations below or equal to those of glucose and IGF-1, or all  
194 four approximately equal and around  $-0.5$  SD. The metabolic cluster was also  
195 considered balanced if all four boxes were inside the  $\pm 0.5$  SD area.

196  
197 *Milk biomarkers*

198 Three different sets of milk biomarkers (MME, FT-MIR spectra and IgG N-glycans)  
199 were considered as predictors. Metabolites and enzymes consisted of six milk  
200 metabolites (glycose-6-phosphate, free glucose, BHB, isocitrate, urea and uric acid)

201 and two enzymes (NAGase and LDH). Fourier transform mid-IR spectra from the 6  
202 farms were standardised into a common format. FT-MIR data consisted of  
203 absorbance values at 212 wavenumbers selected from a total of 1060 by removal of  
204 areas known to be non-reproducible between instruments or non-informative due to  
205 the water component in milk (Grelet et al., 2016). Finally, 19 peaks of IgG *N*-glycans  
206 were manually identified and integrated. Each peak's percentage of the total area  
207 under the 19 peaks was used as the measure for the statistical analyses. Further  
208 details on the laboratory analysis are given in De Koster et al. (2019).

209

### 210 *Random forests predictions*

211 Each of the three sets of milk biomarkers were used to predict the responses (EBAL,  
212 PI-index and metabolic clusters) separately for each parity and period by use of the  
213 random forests algorithm (see below), i.e. in total 54 predictions. In addition, each of  
214 the six plasma metabolites and serum IGF-1 were predicted. To make a more fair  
215 comparison with IgG *N*-glycans, we also made a comparison using only data that  
216 were complete across all three sets of milk biomarkers in relation to the two periods;  
217 around DIM 14 and DIM 35. Random forests belongs to the field of machine learning  
218 and is an ensemble of classification or regression trees (Breiman, 2001) with each  
219 tree being a set of decision rules. A short description of the algorithm is given below,  
220 whereas we refer to Breiman (2001) for a technical presentation and introduction to  
221 random forests. We generally used default settings of the implementation except that  
222 we used 2500 trees (instead of the default 500) to stabilise estimates of accuracy.

223

### 224 *Random forests algorithm*

225 In summary, for each of a pre-specified number of trees (default: 500) a sample is  
226 drawn from the original data by sampling with replacement (bootstrap sample).  
227 These samples have the same size as the original data but contain on average  
228 approximately two thirds of the individual records, since some are selected more than  
229 once and some not at all. Each bootstrap sample is used for training an unpruned  
230 tree. At each node of the tree, a set of predictors (default for binary classification:  
231 square root number of predictors) are chosen at random as candidates for splitting  
232 the data present at the current (parent) node into two chunks. The algorithm then  
233 choose the candidate (categorical) or cut-point (continuous) that give the largest  
234 reduction of the Gini index (Breiman et al., 1984), i.e. the most homogeneous child  
235 nodes. Each tree is grown as large as possible. The random selection of candidate  
236 predictors at each node protects from overfitting (Breiman, 2001) and pruning is not  
237 necessary. When the random forest of trees have been developed, new records are  
238 passed through each tree and majority voting or averaging predicts their classes or  
239 values.

240

#### 241 *Statistical analysis*

242 The statistical analyses were carried out using R version 3.6.1 (R Core Team, 2019).  
243 For k-means clustering the *kmeans* function of R was used. Random forests  
244 modelling was carried out by use of the *randomForest* package (Liaw and Wiener,  
245 2002). We evaluated performance of random forests predictions for metabolic  
246 clusters by a leave-one-cow-out (internal) cross-validation strategy, i.e. in turn  
247 preserving data from one cow as test set and using data from the other cows for  
248 training of a random forests model. By use of the *confusionMatrix* function of the  
249 *caret* package (Kuhn, 2008) we calculated global accuracy (proportion of correctly

250 classified samples, i.e. the diagonal of the 3 by 3 contingency table of predicted  
251 versus true cluster also known as the confusion matrix), sensitivity for each cluster  
252 (proportion correctly predicted to that cluster) and specificity (proportion correctly  
253 predicted not to be in that cluster). In addition, the precision of predictions for the  
254 individual blood biomarkers, EBAL and PI-index was measured by the coefficient of  
255 determination of cross-validation ( $R^2_{cv}$ ) and the root mean squared error ( $RMSE_{cv}$ ).

256

257 To explore the ranking of the individual MME biomarkers within parity and period, the  
258 variable importance measure (VIM) was calculated (Breiman, 2001) and plotted  
259 using *randomForests*. This measure is based on the internal out-of-bag samples, i.e.  
260 the third not picked to be included in each bootstrap sample, see Breiman (2001).

261

262 Characteristics and differences among metabolic clusters in milk metabolite  
263 concentrations, enzyme activities and daily milk yield were examined separately for  
264 parity 2 and 3+ at DIM 14 by ANOVA with F-tests. Since most health events and  
265 imbalances are expected to happen in the first and middle part of the early lactation  
266 period, we only focused on DIM 14 for this part. First parity cows were not given  
267 further attention since none of these were classified to the imbalanced cluster at DIM  
268 14 and all were in clusters classified as balanced at DIM 35.

269

## 270 **Results**

271 Summary statistics for production, blood biomarkers and MME can be found in tables  
272 and supplementary tables of Krogh et al. (2019).

273

274

275 *Predictions of EBAL and PI-index by sets of milk biomarkers*

276 The precisions ( $R^2_{CV}$  and  $RMSE_{CV}$ ) of predicting measures of EBAL and PI-index by  
277 the three sets of milk biomarkers as determined by leave-one-cow-out cross-  
278 validation are shown in Table 1. The best precision was obtained when predicting PI-  
279 index by MME with an  $R^2_{CV}$  of 0.40 at 14 DIM and 0.34 at 35 DIM. For FT-MIR, the  
280 corresponding  $R^2_{CV}$  was 0.26 and 0.19. For EBAL, however, FT-MIR showed a better  
281 performance than MME with an  $R^2_{CV}$  of 0.28 vs 0.21. The RMSEs from MME and FT-  
282 MIR predictions were respectively 23.7 and 23.4 for EBAL and between 1.62 and  
283 1.96 for PI-index. Predictions by IgG N-glycans had the lowest precisions, with  $R^2_{CV}$   
284 ranging between 0.01 and 0.06 and with  $RMSE_{CV}$  being 26.3 for EBAL and 2.04 for  
285 PI-index.

286

287 *Predictions of individual blood biomarkers by sets of milk biomarkers*

288 Predictions of individual blood biomarkers are shown in Table 2. The best precisions  
289 were obtained with MMEs for plasma urea ( $R^2_{CV} = 0.62$  for 14 DIM and 0.59 for 35  
290 DIM) and for plasma BHB ( $R^2_{CV} = 0.46$  and 0.40). Interestingly, plasma cholesterol  
291 was not predicted that well ( $R^2_{CV} = 0.09$  and 0.12) whereas precisions of serum IGF-  
292 1 were at the same level as plasma BHB for DIM 35 ( $R^2_{CV} = 0.40$ ) and a bit lower for  
293 DIM 14 ( $R^2_{CV} = 0.32$ ). The precisions by IgG N-glycans were always the lowest  
294 whereas generally, FT-MIR were at the same level as MME but in some cases much  
295 lower.

296

297 *Metabolic cluster changes*

298 The number of cows in each of the three metabolic clusters at DIM 14 and DIM 35 is  
299 reported in Table 3 with indication of changes between the two periods. All the 52

300 primiparous cows were interpreted balanced at DIM 35. Among the 28 parity 2 cows  
301 in the intermediate cluster at DIM 14, 17 (61%) did not shift to a cluster deemed to be  
302 more "balanced" at DIM35, staying in an intermediate cluster, while the rest changed  
303 to a balanced cluster (N=11). Most of the 23 parity 2 cows in the balanced cluster at  
304 DIM 14 stayed in a balanced cluster at DIM 35 (N=21) with only two cows shifting;  
305 one to an imbalanced and one to an intermediate cluster at DIM 35. For 15 (4+11)  
306 out of 18 (7+11) (83%) parity 2 and 3+ cows in the imbalanced cluster DIM 14, extra  
307 attention may be relevant as they were also in an imbalanced cluster DIM 35.  
308 Concerning parity 3+ cows in the balanced cluster DIM 14, 31 out of 38 (82%) were  
309 still in a balanced cluster at DIM 35 while the rest changed to an imbalanced cluster.  
310 Of the 54 parity 3+ cows in the intermediate cluster DIM 14, 39 (72%) changed to a  
311 balanced cluster at DIM 35, while the rest changed to an imbalanced cluster.

312

### 313 *Prediction of metabolic clusters*

314 Accuracies to predict the clusters from sets of milk biomarkers with random forests  
315 models are presented in Table 4 for each combination of parity (1, 2 and 3+) and  
316 period (DIM 14 and 35). As in Grelet et al. (2019) and De Koster et al. (2019),  
317 including milk yield as a factor in the aim to help distinguishing between classes did  
318 not improve the accuracy (results not shown). Global accuracies from MME and FT-  
319 MIR were at the same level and ranged from 0.54 to 0.65 for MME and 0.51 to 0.68  
320 for FT-MIR. Accuracies were lower for IgG N-glycans; ranging from 0.32 to 0.53. The  
321 sensitivity for prediction of the imbalanced cluster was better with MME than with FT-  
322 MIR and IgG N-glycans. Unfortunately, examples of zero sensitivity (none predicted  
323 correctly) were seen, likely due to a relatively low number of cows in the imbalanced  
324 clusters, see Table 3.

325

326 Results from predictions using only data that were complete across all three sets of  
327 milk biomarkers in each period are shown in Supplementary Table S2 and are less  
328 stable with confidence intervals that are bit wider due to the smaller number of  
329 observations. Nevertheless, predictions by IgG *N*-glycans tend to be less  
330 unfavourable compared to MME and FT-MIR when judged on this reduced data set,  
331 potentially giving a more fair comparison. Global accuracies tended to be lower with  
332 the reduced data set and ranged from 0.39 to 0.59 for MME, 0.34 to 0.67 for FT-MIR  
333 and 0.19 to 0.57 for IgG *N*-glycans. Using this reduced data set, we also examined  
334 the pairwise agreement of predictions among the three sets of milk biomarkers, see  
335 Supplementary Table S3. The best agreement with a global accuracy of 0.76 (95%  
336 CI: 0.62-0.87) was found between MME and FT-MIR for parity 3+ cows around DIM  
337 14 but it should be noted that for these, none of the cows in the imbalanced cluster  
338 were correctly determined by FT-MIR. The lowest agreement was seen between FT-  
339 MIR and IgG *N*-glycans for parity 3+ cows around DIM 35 with a global accuracy of  
340 0.27 (0.16-0.41). Generally, the agreements were at the same level among all three  
341 sets of milk biomarkers.

342

343 To ease comparison with table 6 in Grelet et al. (2019) and figure 5 in De Koster et  
344 al. (2019), we calculated the global accuracy for predicting the imbalanced cluster vs  
345 intermediate and balanced combined. For MME in parity 3+ this accuracy was 0.97  
346 (0.92-0.99) and 0.82 (0.73-0.89) for DIM 14 and 35, respectively. For FT-MIR the  
347 corresponding accuracies were 0.89 (0.81-0.95) and 0.69 (0.59-0.78) and for IgG *N*-  
348 glycans 0.92 (0.82-0.97) and 0.53 (0.40-0.66). These accuracies tend to be higher  
349 DIM 14 and at the same level or lower DIM 35 than those found in Grelet et al. (2019)

350 and De Koster et al. (2019). For parity 2, number of cows in the imbalance clusters  
351 were quite low (see Table 3) and almost all sensitivity estimates were 0 and  
352 specificities at or close to 1 (see Table 4). Thus, parity 2 accuracies are high (e.g.  
353 0.93 (0.83-0.98) for MME at 14 DIM) but driven by specificity.

354

#### 355 *Differences in milk metabolite contents among metabolic clusters*

356 Considering further the characteristics of parity 2 and 3+ cows at DIM 14, Table 5  
357 presents quartiles for milk yield, metabolites and enzymes for each of the three  
358 metabolic clusters. These results indicate that some of the milk metabolites and  
359 enzymes were significantly different between the three metabolic clusters. The  
360 concentration of free glucose was significantly lower in the imbalanced cluster while,  
361 generally, those of BHB and isocitrate were higher. For the parity 2 cows, glucose-6-  
362 phosphate, and free glucose concentrations were higher for the balanced cluster  
363 than for the imbalanced, while for BHB, isocitrate and NAGase the concentrations or  
364 activities were lower or tended ( $P = 0.07$ ) to be lower for the balanced compared to  
365 the imbalanced cluster. For parity 3+ cows, glucose-6-phosphate did not differ  
366 between the metabolic clusters but otherwise the results were similar to those of  
367 second parity cows. For parity 3+ cows, the urea concentration also tended ( $P=0.07$ )  
368 to be higher for the imbalanced cluster compared with the balanced cluster. To  
369 explore the ranking of importance within parity and period for the eight milk  
370 metabolites and enzymes in the MME set of milk biomarkers, VIM plots are shown in  
371 Supplementary Figures S1 to S4. BHB is among the most important for both the 14  
372 and 35 DIM periods whereas isocitrate is important for both parity in the period  
373 around DIM 14 but only for the oldest (3+) cows around DIM 35. For second lactation  
374 cows around DIM 35, free glucose and LDH are marginally more important than BHB

375 which ranks third. For the oldest cows (3+) free glucose is more important than  
376 isocitrate around DIM 14 whereas around DIM 35, uric acid and urea are also  
377 important for the prediction of the metabolic clusters.

378

## 379 **Discussion**

380 The objective was to compare the use of three different sets of milk biomarkers for  
381 identification of cows in physiological imbalance and thus at risk of developing a  
382 metabolic or infectious disease. We defined a metabolic imbalanced cluster of cows  
383 based on k-means clustering of four blood biomarkers; glucose, NEFA and BHB in  
384 plasma and IGF-1 in serum. Random forests was used to predict individual blood  
385 biomarkers, body energy balance (EBAL), index for physiological imbalance (PI-  
386 index) and the clusters differentiating the metabolic status of cows. Ideally, the  
387 prediction algorithms should be validated using an external data set but this was not  
388 possible in the present study. Therefore, internal cross-validation was used to  
389 examine performance.

390

391 IgG *N*-glycans performed really poor compared to the other two sets of milk  
392 biomarkers for predictions of individual blood biomarkers, EBAL, PI-index and  
393 metabolic clusters. This may partly be due to a less dense sampling of this milk  
394 biomarker. Nevertheless, even when accounting for the difference in sampling  
395 density IgG *N*-glycans had lower prediction accuracies than MME, FT-MIR or both. In  
396 addition, the analytical procedure is very complicated, expensive and with large  
397 problems of getting reliable results. Thus, also in that respect more work is needed to  
398 make this milk biomarker useful in herd health management.

399

400 The precision of predictions for the individual blood biomarkers, EBAL and PI-index  
401 was measured by the coefficient of determination of cross-validation ( $R^2_{cv}$ ) and by the  
402 root mean squared error ( $RMSE_{cv}$ ). These two measures of precision were  
403 interpreted with the recommendations from Alexander et al. (2015) in mind that as a  
404 rule of thumb the  $R^2$  should higher than 0.6 and the RMSE within 10% of the  
405 outcome's range.

406

407 To predict individual blood biomarkers, the best models were obtained by MME with  
408  $R^2_{cv}$  of 0.62 and 0.59 for plasma urea at 14 and 35 DIM, respectively. These were  
409 the only predictions reaching the 0.6 threshold mentioned above. Moreover,  $RMSE_{cv}$   
410 for MME predictions (0.72 and 0.78) were below 10% of the plasma urea range at  
411 8.45 mM (supplementary tables of Krogh et al., 2019). The  $R^2_{cv}$  for FT-MIR models  
412 were generally lower than for MME and in some cases much lower, e.g. 0.06 (DIM  
413 14) and 0.13 (DIM 35) for plasma urea. Correspondingly, the  $RMSE_{cv}$  were higher,  
414 e.g. 1.08 and 1.13 for plasma urea at 14 and 35 DIM. Lower performances of the FT-  
415 MIR models, compared to Grelet et al. (2019), may possibly be explained by different  
416 methodologies. In that study all DIM were combined into one global model,  
417 distribution of data were artificially modified and partial least squares regression was  
418 used instead of random forests. These differences were one of the reasons for  
419 redoing the FT-MIR predictions in the present paper.

420

421 For EBAL, FT-MIR showed a better performance than MME with an  $R^2_{cv}$  of 0.28 vs  
422 0.21 whereas the opposite was the case when predicting PI-index with  $R^2_{cv}$  of 0.26  
423 vs 0.40 at 14 DIM and 0.19 vs 0.34 at 35 DIM. Clearly these are below the 0.6 rule of  
424 thumb. The RMSEs from EBAL predictions (23.4 and 26.3) were lower than 10% of

425 the absolute range, whereas for PI-index only RMSEs from MME predictions (1.62  
426 and 1.71) were around 10% of the absolute range.

427

428 Metabolic clusters were created as alternative phenotypes. The global accuracy of  
429 predicting the metabolic clusters varied from 0.54 to 0.65 and 0.51 to 0.68 for MME  
430 and FT-MIR predictions, respectively. Thus, the performance of MME and FT-MIR  
431 was at an equal level. It should be noted that examples of sensitivity at zero and  
432 specificity close to one were seen and may have biased the accuracy upwards.

433 There was no improvement of including daily milk yield in the prediction models, as  
434 also concluded by Ingvarsten et al. (2003). It is not milk yield per se that increases  
435 the risk of diseases but rather physiological imbalance reflecting difficulties for some  
436 animals to adapt to the major physiological changes that occur particularly in the  
437 transition cow. Moreover, this is in accordance with results in Grelet et al. (2019) and  
438 De Koster et al. (2019) though comparison with these two studies is complicated by  
439 differences in examined periods and parities. The present study did notice  
440 differences in blood biomarker profiles among parities but more data would be  
441 desirable for such differentiation. In this study, work has focused on the first 7 weeks  
442 after calving and does not apply to cows at later stages. Since no clusters of  
443 primiparous cows were considered imbalanced, it generally seems from the present  
444 study that first parity cows do not require extra care and the attention should be on  
445 the multiparous cows. Relatively few cows in the imbalance clusters were also  
446 observed for parity 2 accompanied by sensitivity estimates at zero and specificities  
447 close to one. Thus, neither first nor second parity cows were really informative for the  
448 ability to predict the imbalanced cluster.

449

450 The purpose of the presented random forests algorithms were to identify cows in  
451 physiological imbalance at risk of developing subclinical or more severe stages of  
452 diseases. Such cows may need extra attention and potentially altered feeding or  
453 other management actions to avoid that the physiological imbalance develop into  
454 subclinical or more severe disease states. The required accuracy of detection is  
455 obviously lower for this purpose since there is no risk of harm to the animal or of  
456 needless use of medicine. The accuracies mentioned in this paper are likely too low  
457 for diagnosing diseases that require medical treatment with e.g. antibiotics.  
458 Generally, the required accuracy depends on the specific purpose and of e.g.  
459 disease prevalence, costs associated with treatment and possible side-effects. The  
460 required accuracy could be established by simulation methods. Possibly, a larger  
461 data set for training prediction algorithms would improve the accuracies and the  
462 results presented here may be used to guide sample size decisions for future  
463 studies.

464

465 Presently, no sensors are available to measure e.g. free glucose, isocitrate and  
466 glucose-6-phosphate, but since FT-MIR algorithms tended to give as accurate  
467 predictions as MME, FT-MIR may give the same opportunities to make relevant  
468 classification of cows as balanced or in physiological imbalance (see also Grelet et  
469 al., 2019 and De Koster et al., 2019). Moreover, it would also be interesting to  
470 investigate direct prediction of udder inflammation from FT-MIR as opposed to the  
471 use of e.g. LDH and NAGase enzymes that constitute an alternative for somatic cell  
472 counts, helping in the detection of subclinical diseases (Kitchen et al., 1978; Larsen  
473 et al., 2010; Hovinen et al., 2016).

474

475 *Conclusion*

476 Neither EBAL nor PI-index were sufficiently precise to be used as a management tool  
477 for identification of risk cows. As an alternative, cows were divided into clusters  
478 based on measures of glucose, BHB and NEFA in plasma and IGF-1 in serum.  
479 These can be interpreted into metabolic clusters and the cluster of imbalanced cows  
480 can be predicted equally well by MME and FT-MIR. Nevertheless, accuracies still  
481 need to be improved and a larger data set for training the prediction algorithms would  
482 probably be needed. Free glucose, isocitrate, glyucose-6-phosphate, BHB and  
483 NAGase measured in milk were significantly different among the three metabolic  
484 clusters (balanced, intermediate and physiological imbalanced). Thus, if MME is the  
485 preferred set of milk biomarkers to predict cows in physiological imbalance and at  
486 risk of developing a production or infectious disease, the above mentioned  
487 metabolites and enzyme should have high priority for inclusion. The use of IgG N-  
488 glycans for prediction still needs development.

489

490 **Author's contribution**

491 LF, CGa, MAK, MTS and KLI made the first draft of the paper. LF, CGr, MS, MH and  
492 other partners from GC undertook data handling and data quality control. LF, CGr,  
493 MS and MH did the major parts of the statistical analyses including the conception of  
494 the idea of using k-means clusters to combine selected blood biomarkers with  
495 contribution to the latter from MTS and KLI. LF, CGa, MAK, MTS and KLI  
496 collaboratively defined the metabolic interpretation of these clusters. MTS, MAC, KLI  
497 and other partners from GC did the conception and designed the study. TL handled  
498 storage of milk and blood samples and did lab analyses of milk metabolites, milk  
499 enzymes and blood metabolites and assisted during the data quality control of these

500 biomarkers. CGr and other partners from GC undertook analyses and calibrations of  
501 FT-MIR. EM, ROF, FC, MAC and other partners from GC did lab analyses and  
502 interpretation of IgG *N*-glycans. All authors critically revised the first draft and  
503 approved the final version of the manuscript.

504

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511

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513 There is no direct financial interest of the authors and affiliations in the subject matter  
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515

### 516 **Ethics statement**

517 The experiments were carried out in accordance with the standards recommended  
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519

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521 None of the data were deposited in an official repository.

522

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529

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621

622 **Figure captions**

623

624 **Figure 1** Box-and-whiskers plots for graphical interpretation (note that bars are  
625 medians) of k-means clusters into metabolic clusters as indicated by colours:  
626 balanced cluster (magenta), intermediate cluster (orange) and physiological  
627 imbalanced cluster (yellow). Distribution of standardised blood metabolites and IGF-1  
628 in each cluster (1, 2 and 3), at 14 DIM (first row), at 35 DIM (second row), for  
629 primiparous Holstein dairy cows (first column), second parity cows and for parity 3+  
630 cows (last column). The horizontal lines indicate  $\pm 0.5$  SD.

631

632 **Tables**

633

635 **Table 1** Precision of random forests predictions of EBAL and PI-index with three sets  
636 of milk biomarkers (milk metabolites and enzymes (MME), Fourier transform mid-IR  
637 spectra (FT-MIR) and immunoglobulin G (IgG) N-glycans)<sup>1</sup> in Holstein dairy cows in  
638 six herds. The performance was measured by the coefficient of determination of leave-  
639 one-cow-out cross-validation ( $R^2_{cv}$ ) and by root mean squared error ( $RMSE_{cv}$ ).  
640 Individual milk biomarkers were standardised using all available data before matching.  
641 In addition to sets of milk biomarkers, parity (1, 2 and 3+) as a factor and DIM (days in  
642 milk) as continuous covariate were included as predictors for EBAL, whereas only  
643 parity was added as predictor for PI-index. Number of cows (samples) are after  
644 removal of records excluded due to missing values

Response	Period (DIM)	Sets of milk biomarkers	$N_{cows}$ ( $N_{samples}$ )	$R^2_{cv}$	$RMSE_{cv}$
EBAL (only using DK, IE and UK herds)	1-50	MME	132 (1608)	0.21	23.7
		FT-MIR	132 (1230)	0.28	23.4
		IgG	122 (328)	0.06	26.3
PI-index	14	MME	216	0.40	1.62
		FT-MIR	201	0.26	1.86
		IgG	133	0.01	2.04
PI-index	35	MME	218	0.34	1.71
		FT-MIR	195	0.19	1.93
		IgG	134	0.05	2.04

645 <sup>1</sup> Milk biomarkers were matched with the EBAL closest in sampling date (+/- 3 days). For FT-MIR this matching  
646 strategy was also applied to PI-index for the period noted in the column denoted "Period (DIM)". If no perfect match  
647 (same day) was found, we proceeded as follows: Step 1 day backward first (day before milk biomarker sampling  
648 date), then 2 days forward (i.e. 1 day after the sampling data), then 3 days back (corresponding to 2 days before  
649 sampling), then 4 days forward, 5 days backward and 6 days forward. That is, closest match within 7 days (a week)  
650 centred in the milk biomarker's sampling date. For IgG N-glycans, the measure from the period noted was used for  
651 these two measurements. Averages of measures of milk metabolites and enzymes within the same week (Monday-  
652 Sunday) as blood sampling were used for PI-index.  
653

654 **Table 2** Precision ( $R^2$  and RMSE by leave-one-cow-out cross-validation) of random  
655 forests predictions of plasma metabolites and serum IGF-1 with three sets of milk  
656 biomarkers (milk metabolites and enzymes (MME), Fourier transform mid-IR spectra  
657 (FT-MIR) and immunoglobulin G (IgG) N-glycans) in Holstein dairy cows. Individual  
658 milk biomarkers were standardised and the sample matching the blood sample date  
659 (+/- 3 days) was used. In addition, parity (1, 2 and 3+) was included as a predictor.  
660 Number of cows are after removal of those excluded due to missing values

Blood biomarker	Period (DIM)	Sets of milk biomarkers	N <sub>cows</sub>	R <sup>2</sup> <sub>cv</sub>	RMSE <sub>cv</sub>
Plasma fructosamine	14	MME	213	0.12	16.9
		FT-MIR	198	0.11	17.2
		IgG	131	0.03	17.6
	35	MME	214	0.18	16.4
		FT-MIR	191	0.02	18.5
		IgG	132	0.11	17.2
Plasma urea	14	MME	216	0.62	0.72
		FT-MIR	201	0.06	1.08
		IgG	133	0.01	1.07
	35	MME	218	0.59	0.78
		FT-MIR	195	0.13	1.13
		IgG	134	0.01	1.16
Plasma cholesterol	14	MME	216	0.09	0.68
		FT-MIR	201	0.01	0.72
		IgG	133	0.01	0.72
	35	MME	218	0.12	0.98
		FT-MIR	195	0.03	1.02
		IgG	134	0.04	1.02
Plasma log <sub>10</sub> (NEFA)	14	MME	216	0.13	0.25
		FT-MIR	201	0.10	0.26
		IgG	133	<0.01	0.26
	35	MME	218	0.09	0.30
		FT-MIR	195	0.03	0.31
		IgG	134	0.01	0.32
Plasma glucose	14	MME	216	0.29	0.41
		FT-MIR	201	0.23	0.43
		IgG	133	0.11	0.49
	35	MME	218	0.32	0.43
		FT-MIR	195	0.19	0.48
		IgG	134	0.17	0.49
Plasma log <sub>10</sub> (BHB)	14	MME	216	0.46	0.16
		FT-MIR	201	0.27	0.20
		IgG	133	0.04	0.24
	35	MME	218	0.40	0.17
		FT-MIR	195	0.25	0.19
		IgG	134	<0.01	0.22
Serum log <sub>10</sub> (IGF-1)	14	MME	216	0.32	0.27
		FT-MIR	204	0.36	0.26
		IgG	136	0.24	0.29
	35	MME	216	0.40	0.21
		FT-MIR	197	0.35	0.22
		IgG	138	0.14	0.25

662 **Table 3** *Number of Holstein dairy cows per metabolic cluster (balanced,*  
663 *intermediate, imbalanced) at DIM 14 and 35. Furthermore, the last column shows*  
664 *which clusters the DIM 35 cows belonged to at DIM 14*

Cluster and parity	Number of cows		Cluster affiliation at DIM 14 for DIM 35 cows
	DIM 14	DIM 35	
<i>Parity 1</i>			
Balanced	38	52	38 Balanced + 14 Intermediate
Intermediate	14	0	
Imbalanced	0	0	
<i>Parity 2</i>			
Balanced	23	32	21 Balanced + 11 Intermediate
Intermediate	28	21	1 Balanced +17 Intermediate + 3 Imbalanced
Imbalanced	7	5	1 Balanced + 4 Imbalanced
<i>Parity 3+</i>			
Balanced	38	70	31 Balanced + 39 Intermediate
Intermediate	54	0	
Imbalanced	11	33	7 Balanced +15 Intermediate + 11 Imbalanced
Total	213	213	

665

666 **Table 4** Leave-one-cow-out cross-validation of performance for random forests predictions of metabolic clusters by milk metabolites  
667 and enzymes (MME), Fourier transform mid-IR (FT-MIR) spectra and immunoglobulin G (IgG) N-glycans. Clusters based on k-means  
668 clustering ( $k=3$ ) of standardised values of plasma glucose,  $\log_{10}(\text{BHB})$  and  $\log_{10}(\text{NEFA})$  and serum  $\log_{10}(\text{IGF-1})$  in Holstein dairy cows

Period and parity	Cluster number <sup>1</sup>	Metabolic cluster <sup>2</sup>	Sensitivity			Specificity			Global accuracy <sup>3</sup> (95% CI)		
			MME	FT-MIR	IgG	MME	FT-MIR	IgG	MME	FT-MIR	IgG
Parity 1											
DIM 14	1	Balanced	0.74	0.70	0.38	0.52	0.61	0.48	0.54	0.51	0.32
	2	Balanced	0.14	0.40	0.10	0.89	0.75	0.79	(0.39-0.68)	(0.37-0.65)	(0.17-0.51)
	3	Intermediate	0.60	0.31	0.45	0.84	0.87	0.70			
DIM 35	1	Balanced	0.63	0.25	0.00	0.98	0.90	1.00	0.62	0.68	0.43
	2	Balanced	0.68	0.83	0.69	0.63	0.71	0.21	(0.47-0.75)	(0.53-0.81)	(0.25-0.63)
	3	Balanced	0.53	0.69	0.18	0.73	0.87	0.68			
Parity 2											
DIM 14	1	Imbalanced	0.50	0.00	0.00	0.98	0.98	1.00	0.55	0.59	0.46
	2	Balanced	0.50	0.70	0.42	0.68	0.65	0.70	(0.42-0.68)	(0.45-0.72)	(0.29-0.63)
	3	Intermediate	0.61	0.70	0.61	0.53	0.68	0.29			
DIM 35	1	Imbalanced	0.00	0.00	0.00	0.98	0.96	1.00	0.58	0.55	0.53
	2	Balanced	0.79	0.69	0.71	0.50	0.52	0.53	(0.44-0.70)	(0.40-0.69)	(0.35-0.70)
	3	Intermediate	0.36	0.50	0.44	0.70	0.71	0.60			
Parity 3+											
DIM 14	1	Imbalanced	0.70	0.00	0.00	1.00	0.99	1.00	0.63	0.66	0.51
	2	Intermediate	0.74	0.76	0.74	0.51	0.63	0.17	(0.53-0.73)	(0.56-0.76)	(0.38-0.64)
	3	Balanced	0.46	0.70	0.17	0.78	0.76	0.74			
DIM 35	1	Imbalanced	0.71	0.59	0.10	0.87	0.74	0.73	0.65	0.59	0.44
	2	Balanced	0.71	0.63	0.71	0.68	0.82	0.70	(0.55-0.75)	(0.49-0.70)	(0.31-0.57)
	3	Balanced	0.50	0.56	0.45	0.90	0.83	0.73			

669 <sup>1</sup> The cluster numbers are arbitrary and cannot be compared among period/parity combinations.

670 <sup>2</sup> As interpreted from Figure 1. The metabolic clusters are comparable among period/parity combinations.

671 <sup>3</sup> Proportion of correctly classified observations by the prediction, i.e. the diagonal of the confusion matrix.

672 **Table 5** Characteristics<sup>1</sup> of milk yield, metabolites and enzymes and comparisons among the  
 673 three metabolic clusters (balanced, intermediate and physiological imbalanced) of Holstein dairy  
 674 cows at DIM 14 in parity 2 and 3+, respectively. Results of ANOVA F-tests for differences among  
 675 metabolic clusters are indicated<sup>2</sup>

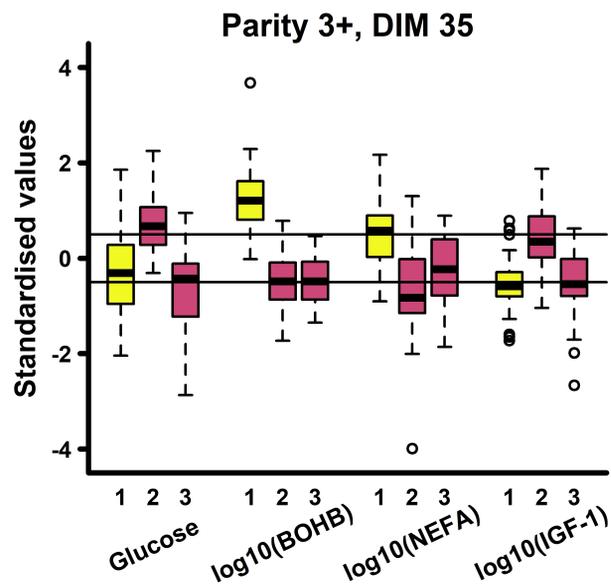
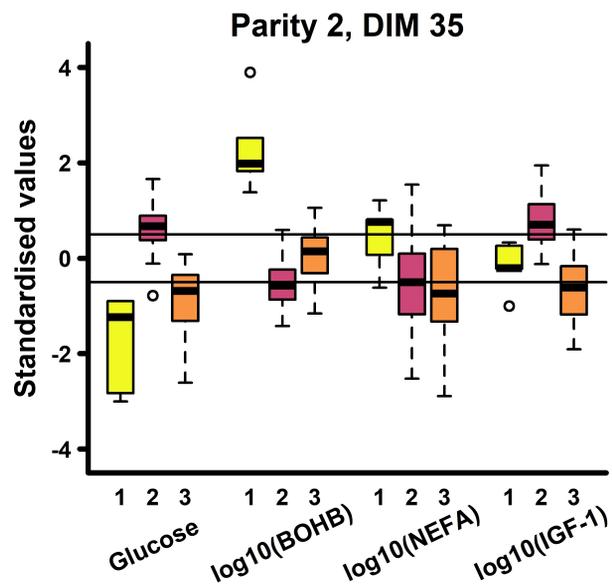
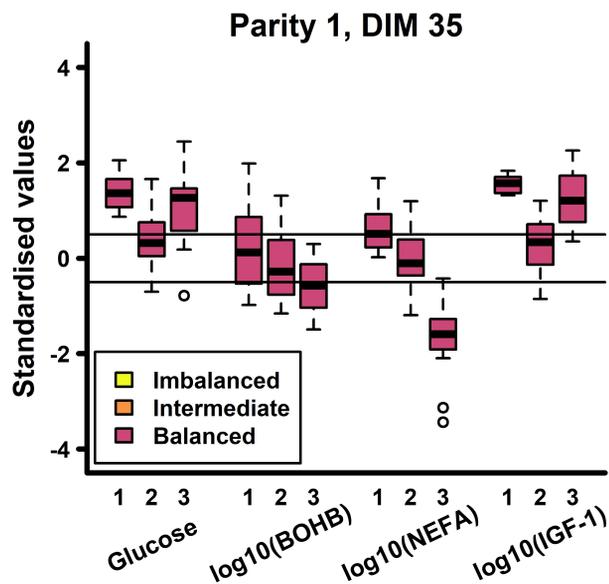
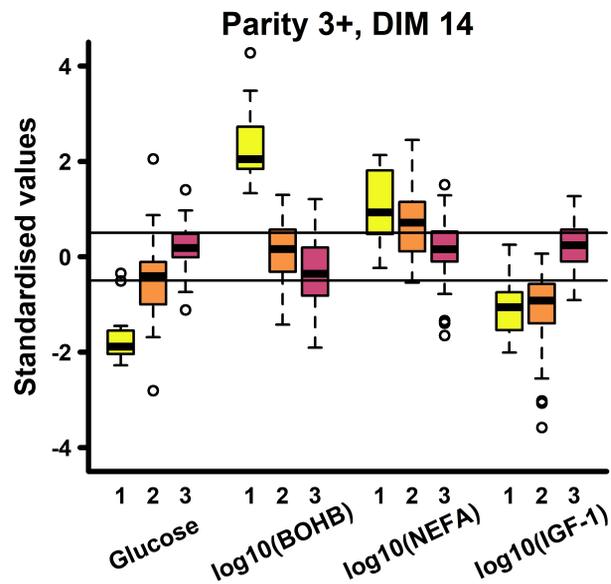
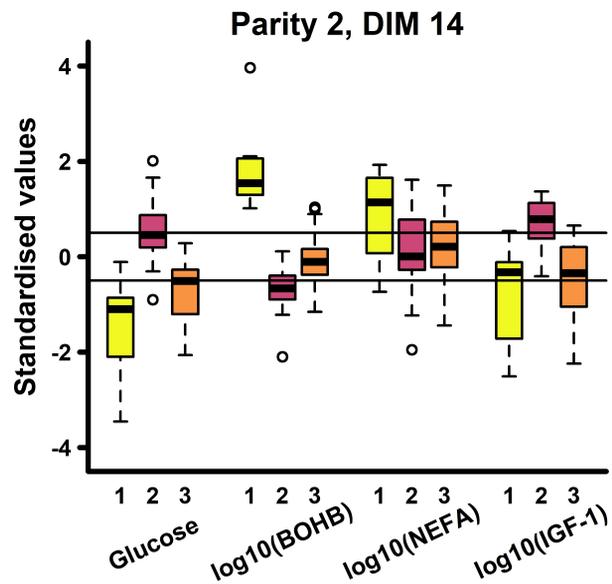
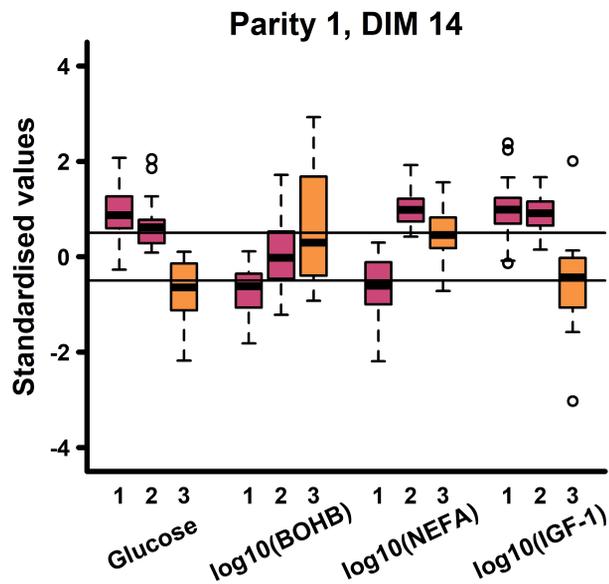
Milk measure and parity	Balanced (n=24) <sup>4</sup>			Intermediate (n=28)			Imbalanced (n=9) <sup>4</sup>			
	Q1	Q2	Q3	Q1	Q2	Q3	Q1	Q2	Q3	
Parity 2										
Glucose-6-P (mM)	0.17	0.22	0.28	0.14	0.18	0.20	0.16	0.18	0.23	*
Free glucose (mM)	0.18	0.25	0.28	0.17	0.22	0.26	0.07	0.12	0.15	**
log <sub>10</sub> (BHB) <sup>3</sup>	1.56	1.63	1.72	1.66	1.76	1.85	1.98	2.06	2.40	***
Isocitrate (mM)	0.15	0.17	0.19	0.17	0.19	0.20	0.19	0.28	0.29	**
Urea (mM)	2.47	3.15	3.83	2.16	3.18	3.79	2.66	2.82	4.90	ns
Uric acid (μM)	161	176	204	154	164	203	139	173	181	ns
log <sub>10</sub> (NAGase) <sup>3</sup>	0.24	0.35	0.46	0.18	0.26	0.41	0.41	0.42	0.46	ns
log <sub>10</sub> (LDH) <sup>3</sup>	0.37	0.46	0.63	0.42	0.56	0.68	0.46	0.57	0.72	ns
Milk yield (kg/day)	30.5	32.4	36.8	26.3	31.6	35.9	28.2	30.5	34.4	ns
Parity 3+										
	Balanced (n=39) <sup>4</sup>			Intermediate (n=54)			Imbalanced (n=11)			
Glucose-6-P (mM)	0.15	0.19	0.24	0.15	0.17	0.22	0.16	0.18	0.20	ns
Free glucose (mM)	0.17	0.21	0.24	0.13	0.16	0.18	0.09	0.10	0.11	***
log <sub>10</sub> BHB <sup>3</sup>	1.55	1.66	1.74	1.66	1.74	1.92	2.05	2.12	2.23	***
Isocitrate (mM)	0.14	0.16	0.19	0.15	0.18	0.21	0.22	0.26	0.28	***
Urea (mM)	2.26	3.12	3.63	1.87	2.76	3.57	2.96	3.17	4.62	ns
Uric acid (μM)	126	166	200	114	155	187	144	174	203	ns
log <sub>10</sub> (NAGase) <sup>3</sup>	0.17	0.27	0.36	0.24	0.35	0.47	0.48	0.55	0.62	**
log <sub>10</sub> (LDH) <sup>3</sup>	0.28	0.41	0.61	0.38	0.48	0.67	0.55	0.64	0.73	ns
Milk yield (kg/day)	34.3	36.4	40.6	32.1	34.6	38.6	29.9	33.0	36.7	ns

676 <sup>1</sup> Q1: first quartile, Q2: second quartile (median), Q3: third quartile, M: molar (mol/L).

677 <sup>2</sup> ns P≥0.05; \* P<0.05; \*\* P<0.01; \*\*\* P<0.001

678 <sup>3</sup> BHB (μM), NAGase (units/L), LDH (units/L).

679 <sup>4</sup> The difference in totals compared to Table 3 is due to cows only having measures DIM 14.



## **Predicting physiological imbalance in Holstein dairy cows by three different sets of milk biomarkers**

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**Supplementary Table S1.** *Number of Holstein dairy cows (row proportion) and summary statistics of parity (mean, SD, median and maximum) for each combination of parity, herd and diet, and pooled*

Herd <sup>1</sup>	Diet <sup>2</sup>	Parity			Total	Mean (SD); median; max
		1	2	3+		
UK	Low C	6 (0.30)	4 (0.20)	10 (0.50)	20	2.6 (1.5); 2.5; 7
	Standard C	6 (0.30)	2 (0.10)	12 (0.60)	20	2.9 (1.6); 3; 6
	High C	6 (0.29)	3 (0.14)	12 (0.57)	21	2.8 (1.6); 3; 7
	Pooled	18 (0.30)	9 (0.15)	34 (0.56)	61	2.7 (1.6); 3; 7
DK	High starch	5 (0.45)	2 (0.18)	4 (0.36)	11	2.5 (1.8); 2; 5
	High sugar	4 (0.40)	3 (0.30)	3 (0.30)	10	2.5 (1.8); 2; 6
	Standard	2 (0.14)	9 (0.64)	3 (0.21)	14	2.1 (0.6); 2; 3
	Pooled	11 (0.31)	14 (0.40)	10 (0.29)	35	2.3 (1.4); 2; 6
IE	Standard	2 (0.06)	11 (0.31)	23 (0.64)	36	3.3 (1.5); 3; 7
BE	Standard	13 (0.42)	9 (0.29)	9 (0.29)	31	2.3 (1.6); 2; 6
DE	Standard	3 (0.12)	8 (0.31)	15 (0.58)	26	2.5 (0.7); 3; 3
IT	Standard	8 (0.18)	15 (0.33)	22 (0.49)	45	2.6 (1.2); 2; 6
All	Pooled	55 (0.24)	66 (0.28)	113 (0.48)	234	2.6 (1.4); 2; 7

<sup>1</sup> UK (Agri-Food and Biosciences Institute, Northern Ireland, UK); DK (Aarhus University, Denmark); IE (UCD Lyons Research Farm, University College Dublin, Ireland); BE (Walloon Agricultural Research Centre, Belgium); DE (Leibniz Institute for Farm Animal Biology, Germany) and IT (Consiglio per la Ricerca in Agricoltura, Italy).

<sup>2</sup> C=concentrate.

**Supplementary Table S2** Leave-one-cow-out cross-validation of prediction performance for milk metabolites and enzymes (MME), Fourier transform mid-IR spectra (FT-MIR) and immunoglobulin G (IgG) N-glycans predictions of metabolic clusters based on k-means clustering ( $k=3$ ) of standardised values of plasma glucose, plasma  $\log_{10}$ (BHB), plasma  $\log_{10}$ (NEFA), and serum  $\log_{10}$ (IGF-1) in Holstein dairy cows. Data with the restriction that all three milk biomarkers were successfully measured in the period

Period and parity	Cluster number <sup>1</sup>	Metabolic cluster <sup>2</sup>	Sensitivity			Specificity			Global accuracy <sup>3</sup> (95% CI)		
			MME	FT-MIR	IgG	MME	FT-MIR	IgG	MME	FT-MIR	IgG
Parity 1											
DIM 14	1	Balanced	0.62	0.69	0.38	0.39	0.53	0.16			
	2	Balanced	0.00	0.10	0.00	0.82	0.73	0.64	0.39	0.34	0.19
	3	Intermediate	0.44	0.11	0.11	0.82	0.74	0.91	(0.22-0.58)	(0.19-0.53)	(0.07-0.36)
DIM 35	1	Balanced	0.00	0.00	0.00	1.00	0.92	1.00			
	2	Balanced	0.79	0.86	0.57	0.46	0.77	0.31	0.56	0.67	0.41
	3	Balanced	0.40	0.60	0.30	0.71	0.76	0.59	(0.35-0.75)	(0.46-0.83)	(0.22-0.61)
Parity 2											
DIM 14	1	Imbalanced	0.00	0.00	0.00	0.96	0.96	1.00			
	2	Balanced	0.33	0.75	0.42	0.63	0.71	0.76	0.39	0.67	0.48
	3	Intermediate	0.50	0.81	0.69	0.27	0.76	0.29	(0.22-0.58)	(0.48-0.82)	(0.31-0.66)
DIM 35	1	Imbalanced	0.00	0.33	0.00	1.00	0.96	1.00			
	2	Balanced	0.64	0.64	0.45	0.59	0.71	0.82	0.50	0.61	0.57
	3	Intermediate	0.50	0.64	0.79	0.50	0.64	0.36	(0.31-0.69)	(0.41-0.79)	(0.37-0.76)
Parity 3+											
DIM 14	1	Imbalanced	0.00	0.00	0.00	1.00	1.00	1.00			
	2	Intermediate	0.85	0.76	0.70	0.19	0.50	0.32	0.59	0.65	0.53
	3	Balanced	0.24	0.61	0.33	0.86	0.78	0.70	(0.45-0.72)	(0.51-0.78)	(0.39-0.66)
DIM 35	1	Imbalanced	0.50	0.00	0.12	0.79	0.71	0.87			
	2	Balanced	0.47	0.58	0.63	0.66	0.64	0.78	0.56	0.35	0.45
	3	Balanced	0.68	0.42	0.58	0.89	0.67	0.53	(0.41-0.69)	(0.22-0.49)	(0.32-0.59)

<sup>1</sup> The cluster numbers are arbitrary and cannot be compared among period/parity combinations.

<sup>2</sup> As interpreted from Figure 1. The metabolic clusters are comparable among period/parity combinations.

<sup>3</sup> Proportion of correctly classified observations by the prediction, i.e. diagonal of the confusion matrix.

**Supplementary Table S3** *Pairwise comparisons of agreement by leave-one-cow-out cross-validation among milk metabolites and enzymes (MME), Fourier transform mid-IR spectra (FT-MIR) and immunoglobulin G (IgG) N-glycans for prediction of metabolic clusters based on k-means clustering (k=3) of standardised values of plasma glucose, plasma log<sub>10</sub>(BHB), plasma log<sub>10</sub>(NEFA), and serum log<sub>10</sub>(IGF-1) in Holstein dairy cows. Data with the restriction that all three milk biomarkers were successfully measured in the period*

Period and parity	Cluster number <sup>1</sup>	Metabolic cluster <sup>2</sup>	Sensitivity			Specificity			Global accuracy <sup>3</sup> (95% CI)		
			MME / FT-MIR	MME / IgG	FT-MIR / IgG	MME / FT-MIR	MME / IgG	FT-MIR / IgG	MME / FT-MIR	MME / IgG	FT-MIR / IgG
Parity 1											
DIM 14	1	Balanced	0.76	0.65	0.52	0.57	0.45	0.36			
	2	Balanced	0.14	0.25	0.13	0.88	0.91	0.75	0.55	0.52	0.38
	3	Intermediate	0.43	0.33	0.00	0.79	0.75	0.76	(0.36-0.73)	(0.33-0.70)	(0.21-0.56)
DIM 35	1	Balanced	0.00	<sup>4</sup>	<sup>4</sup>	1.00	1.00	0.93			
	2	Balanced	0.73	0.53	0.53	0.42	0.10	0.40	0.56	0.37	0.48
	3	Balanced	0.40	0.10	0.40	0.71	0.53	0.65	(0.35-0.75)	(0.19-0.58)	(0.29-0.68)
Parity 2											
DIM 14	1	Imbalanced	0.00	<sup>4</sup>	<sup>4</sup>	0.97	0.97	0.97			
	2	Balanced	0.31	0.30	0.50	0.61	0.62	0.57	0.42	0.48	0.52
	3	Intermediate	0.53	0.57	0.52	0.29	0.30	0.50	(0.25-0.61)	(0.30-0.67)	(0.34-0.69)
DIM 35	1	Imbalanced	0.00	<sup>4</sup>	<sup>4</sup>	1.00	1.00	0.93			
	2	Balanced	0.58	0.63	0.75	0.56	0.55	0.70	0.54	0.57	0.64
	3	Intermediate	0.57	0.55	0.60	0.57	0.63	0.75	(0.34-0.72)	(0.37-0.76)	(0.44-0.81)
Parity 3+											
DIM 14	1	Imbalanced	<sup>4</sup>	<sup>4</sup>	<sup>4</sup>	1.00	1.00	1.00			
	2	Intermediate	0.94	0.81	0.63	0.39	0.12	0.29	0.76	0.59	0.53
	3	Balanced	0.39	0.12	0.29	0.94	0.81	0.63	(0.62-0.87)	(0.45-0.72)	(0.39-0.66)
DIM 35	1	Imbalanced	0.27	0.29	0.14	0.70	0.70	0.79			
	2	Balanced	0.29	0.47	0.25	0.53	0.66	0.46	0.30	0.39	0.27
	3	Balanced	0.32	0.36	0.32	0.69	0.73	0.59	(0.18-0.44)	(0.26-0.53)	(0.16-0.41)

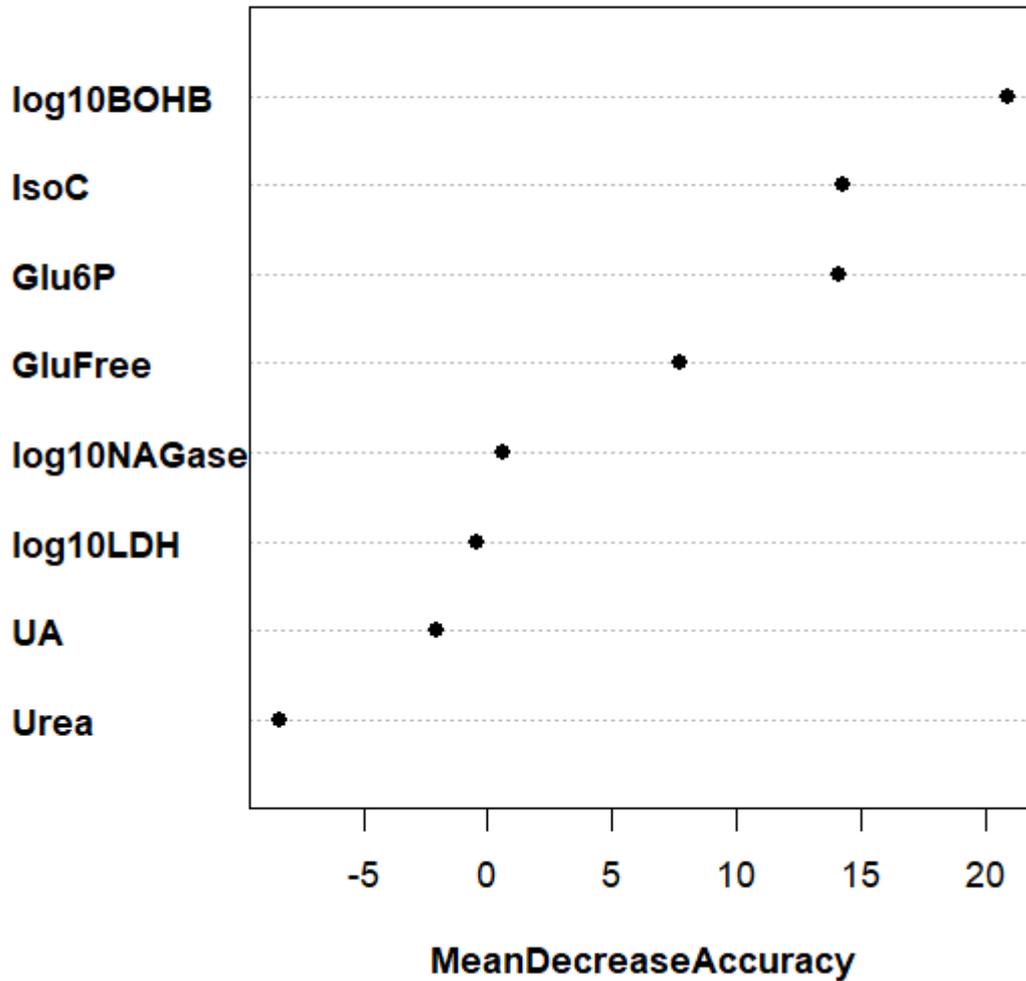
<sup>1</sup> The cluster numbers are arbitrary and cannot be compared among period/parity combinations.

<sup>2</sup> As interpreted from Figure 1. The metabolic clusters are comparable among period/parity combinations.

<sup>3</sup> Proportion of predictions that are the same between methods, i.e. diagonal of the confusion matrix.

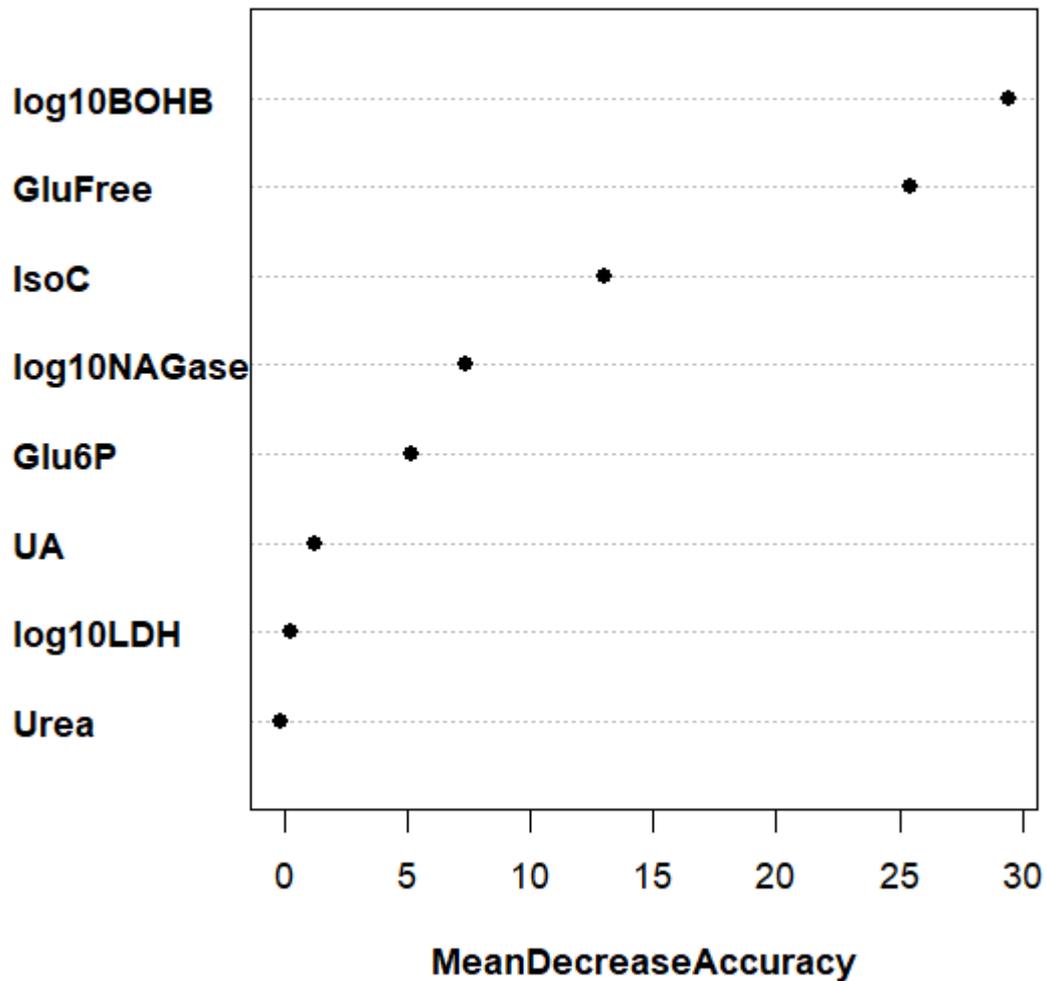
<sup>4</sup> None predicted in the cluster by the "reference" milk biomarker (last mentioned, e.g. IgG).

**Random forests VIM for k-means  
(k=3) clusters from 2 parity cows in DIM14  
pred. by milk metabolites (and Parity)**



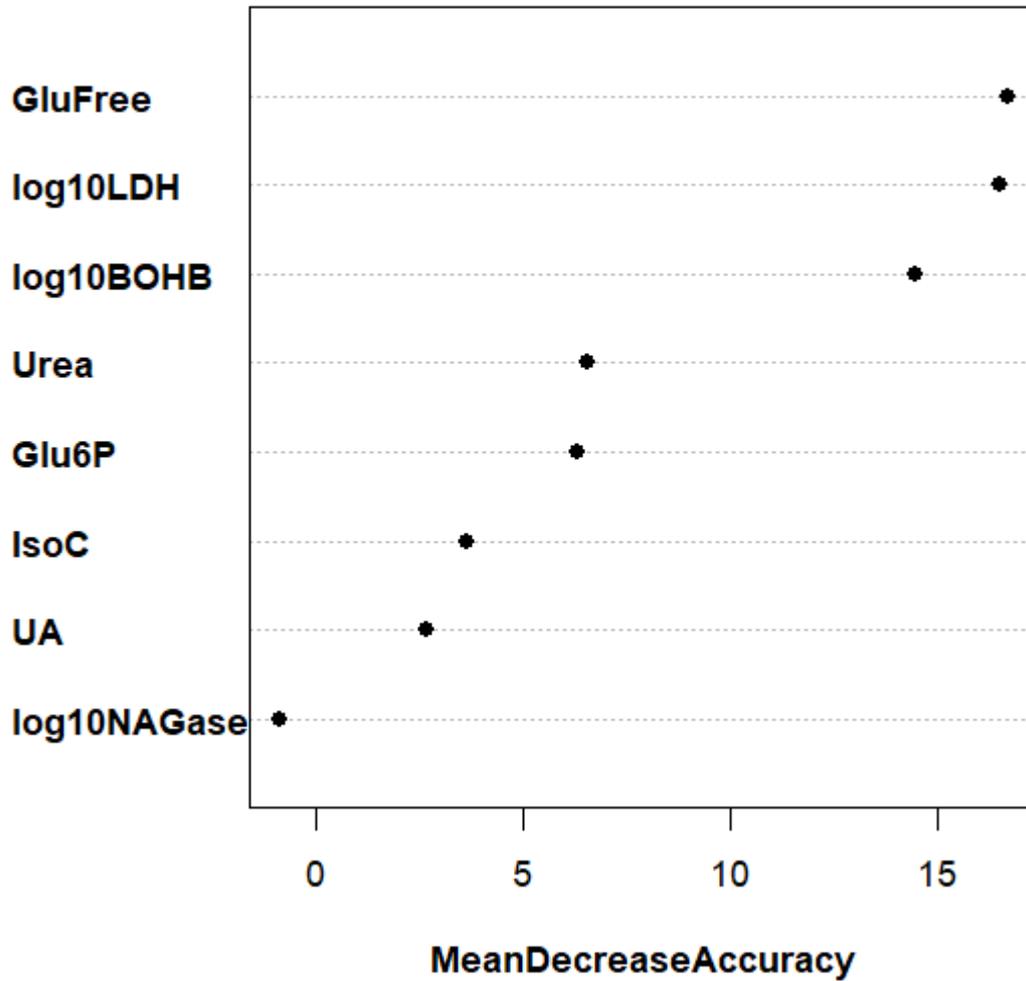
**Supplementary Figure S1** Plot of the variable importance measure (VIM) from the random forests algorithm predicting metabolic clusters by a milk biomarker set of eight milk metabolites and enzymes measured around 14 days after calving (DIM14) in second parity cows.

**Random forests VIM for k-means  
(k=3) clusters from 3+ parity cows in DIM14  
pred. by milk metabolites (and Parity)**



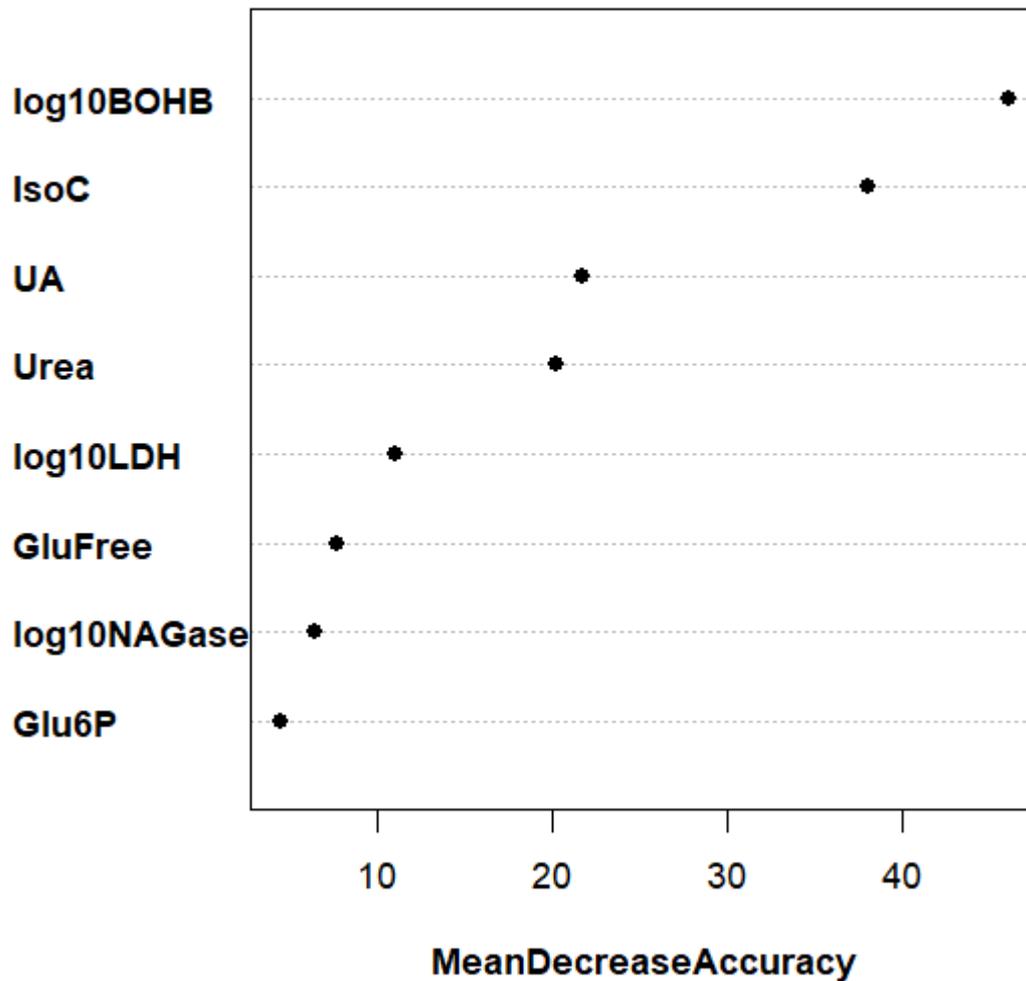
**Supplementary Figure S2** Plot of the variable importance measure (VIM) from the random forests algorithm predicting metabolic clusters by a milk biomarker set of eight milk metabolites and enzymes measured around 14 days after calving (DIM14) in cows with three or more lactations (parity 3+).

**Random forests VIM for k-means  
(k=3) clusters from 2 parity cows in DIM35  
pred. by milk metabolites (and Parity)**



**Supplementary Figure S3** Plot of the variable importance measure (VIM) from the random forests algorithm predicting metabolic clusters by a milk biomarker set of eight milk metabolites and enzymes measured around 35 days after calving (DIM35) in second parity cows.

**Random forests VIM for k-means  
(k=3) clusters from 3+ parity cows in DIM35  
pred. by milk metabolites (and Parity)**



**Supplementary Figure S4** Plot of the variable importance measure (VIM) from the random forests algorithm predicting metabolic clusters by a milk biomarker set of eight milk metabolites and enzymes measured around 35 days after calving (DIM35) in cows with three or more lactations (parity 3+).