



Prediction of detailed blood metabolic profile using milk infrared spectra and machine learning methods in dairy cattle

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ABSTRACT

The adoption of preventive management decisions is crucial to dealing with metabolic impairments in dairy cattle. Various serum metabolites are known to be useful indicators of the health status of cows. In this study, we used milk Fourier-transform mid-infrared (FTIR) spectra and various machine learning (ML) algorithms to develop prediction equations for a panel of 29 blood metabolites, including those related to energy metabolism, liver function/hepatic damage, oxidative stress, inflammation/innate immunity, and minerals. For most traits, the data set comprised observations from 1,204 Holstein-Friesian dairy cows belonging to 5 herds. An exception was represented by β -hydroxybutyrate prediction, which contained observations from 2,701 multibreed cows pertaining to 33 herds. The best predictive model was developed using an automatic ML algorithm that tested various methods, including elastic net, distributed random forest, gradient boosting machine, artificial neural network, and stacking ensemble. These ML predictions were compared with partial least squares regression, the most commonly used method for FTIR prediction of blood traits. Performance of each model was evaluated using 2 cross-validation (CV) scenarios: 5-fold random (CV_r) and herd-out (CV_h). We also tested the best model's ability to classify values precisely in the 2 extreme tails, namely, the 25th (Q25) and 75th (Q75) percentiles (true-positive prediction scenario). Compared with partial least squares regression, ML algorithms achieved more accurate performance. Specifically, elastic net increased the R^2 value from 5% to 75% for CV_r and 2% to 139% for CV_h, whereas the stacking ensemble increased the R^2 value from 4% to 70% for CV_r and 4% to 150% for CV_h. Considering the best model, with the CV_r scenario, good predic-

tion accuracies were obtained for glucose ($R^2 = 0.81$), urea ($R^2 = 0.73$), albumin ($R^2 = 0.75$), total reactive oxygen metabolites ($R^2 = 0.79$), total thiol groups ($R^2 = 0.76$), ceruloplasmin ($R^2 = 0.74$), total proteins ($R^2 = 0.81$), globulins ($R^2 = 0.87$), and Na ($R^2 = 0.72$). Good prediction accuracy in classifying extreme values was achieved for glucose (Q25 = 70.8%, Q75 = 69.9%), albumin (Q25 = 72.3%), total reactive oxygen metabolites (Q25 = 75.1%, Q75 = 74%), thiol groups (Q75 = 70.4%), total proteins (Q25 = 72.4%, Q75 = 77.2%), globulins (Q25 = 74.8%, Q75 = 81.5%), and haptoglobin (Q75 = 74.4%). In conclusion, our study shows that FTIR spectra can be used to predict blood metabolites with relatively good accuracy, depending on trait, and are a promising tool for large-scale monitoring.

Key words: milk spectra, blood metabolites, machine learning

INTRODUCTION

The adoption of preventive management decisions is crucial to dealing with metabolic disorders in dairy cattle. Subclinical metabolic dysfunctions, which occur without clinical signs, are of concern in lactating dairy cows because of their significant effect on health and performance, and subsequent major economic losses at the herd level (Suthar et al., 2013; Sundrum, 2015).

The metabolic health and nutritional status of cows are usually monitored with a well-established analysis known as blood metabolic profiling (Payne et al., 1970; Puppel and Kuczyńska, 2016). This type of analysis is not necessarily performed to identify animals with overt disease, but rather to determine the nutritional and metabolic status of a clinically healthy herd to estimate the prevalence and risk thresholds of subclinical metabolic impairments (Calamari et al., 2016; Luke et al., 2019). Among the various hematochemical parameters that may be analyzed in the blood metabolic profile, nonesterified fatty acids (NEFA) and BHB are recognized and extensively explored indicators of

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metabolic impairments and are commonly used for diagnostics during early lactation (Oetzel, 2004). A body of research has pointed out that immune-metabolic changes can start during late lactation and the dry-off period and have long-term carryover effects postcalving and after lactation (Duske et al., 2009; Caixeta and Omontese, 2021). In this regard, there is the need to deepen the knowledge of indicators that could span throughout the lactation period, monitoring side effects of metabolic changes such as hepatic overload, systemic inflammation, and oxidative stress status. Other metabolites such as liver function enzymes (e.g., γ -glutamyl transferase and aspartate aminotransferase), immunity and inflammation metabolites including negative and positive acute-phase proteins, products of oxidation (e.g., reactive oxygen metabolites), and antioxidant compounds are relatively unexploited, alone or in combination, as potential distress indicators, and have been showed to be informative regarding cow metabolic adaptation throughout the entire lactation period (Premi et al., 2021; Andjelić et al., 2022).

Although there are evident advantages to metabolic profiling, blood sampling on a regular basis remains an invasive and labor-intensive procedure (Aernouts et al., 2020). Furthermore, laboratory analyses of comprehensive metabolic panels are costly and time-consuming (Sailer et al., 2018). In contrast, milk sampling is non-invasive and can be incorporated easily into current standard milking procedures. The detailed composition of raw milk reflects the health and nutritional status of dairy cows, whereas the disruption of metabolic homeostasis is manifested by alterations in the components (Overton et al., 2017; Gross and Bruckmaier, 2019). For instance, MUN is used by nutritionists to monitor dietary protein intake and N use (Hof et al., 1997), and variations in the milk fat-to-lactose and milk fat-to-protein ratios have been found to be associated with metabolic and inflammatory disease onset (Paudyal et al., 2016).

Fourier-transform mid-infrared (FTIR) spectroscopy of milk is widely used as a cost-effective means of assessing the composition of raw milk (Grelet et al., 2015). It is noteworthy that when milk spectra are stored, they can be used any time later to predict new phenotypes, provided they are standardized properly (Soyeurt et al., 2020). With a view to reducing resource-intensive analyses, in recent years several studies have used milk mid-infrared spectroscopy for the estimation of not only those elements dissolved in milk that are health indicators (Grelet et al., 2016), but also metabolites in the blood (Pralle and White, 2020). Using various statistical algorithms, such as partial least squares (PLS) regression and machine learning (ML), satis-

factory prediction equations have been developed from relatively large populations of cows for urea, glucose, total cholesterol (Benedet et al., 2019; Grelet et al., 2019; Luke et al., 2019), NEFA, and BHB (Pralle et al., 2018; Aernouts et al., 2020; Ho et al., 2021).

However, these studies focused on a small number of blood indicators, which therefore only capture in part the complexity of, and the wide variations in, nutritional and metabolic processes. The predictive ability of milk FTIR spectra for a wider range of well-established blood metabolites associated with variations in individual metabolic status has not yet been investigated.

In this study, we explored various ML methods in combination with milk FTIR spectral data to develop prediction equations for a complete blood metabolic profile comprising 29 blood metabolites related to energy metabolism, liver function/hepatic damage, oxidative stress and inflammation/innate immunity, and minerals. Then, we compared the performance of ML methods with that of PLS regression. Last, using the best method for each metabolite, we also assessed the ability to classify values accurately in the 2 extreme tails of the distribution of blood metabolites [25th and 75th percentiles (**Q25** and **Q75**, respectively)], because individuals included in these ranges could represent animals at risk of developing disease.

MATERIALS AND METHODS

Herd and Animal Data

This study used data collected from different research projects: BENELAT and LATSAN, which are aimed at developing new strategies to improve dairy farming animal welfare and milk quality (Pegolo et al., 2021, 2022), and Cowplus, which investigated multibreed dairy production systems in mountain areas (Stocco et al., 2017). As reported in the aforementioned literature, blood metabolites and milk FTIR spectra were acquired using the same methodology within the same laboratory. The research was approved by the Committee for the Protection and Welfare of Experimental Animals (OPBA – Organismo Preposto al Benessere degli Animali) of the Catholic University of the Sacred Heart and by the Italian Ministry of Health (protocol number 510/2019-PR of 19/07/2019).

The final data set included observations from 2,741 dairy cows from 33 herds reared in semi-intensive management systems and located in various regions of northern Italy (Table 1). Farms 1 to 5 reared Holstein-Friesian cows, whereas farms 6 to 33 reared multibreed herds of 6 different breeds: Holstein-Friesian, Brown Swiss, Jersey, Simmental, Rendena, and Alpine Grey.

Table 1. Schematic representation of different sources of information exploited in this study

Herd	Breed	Location (Italy)	Phenotypes			
			Milk yield, kg/d	Complete blood profile ¹	BHB	FTIR spectra ²
1	Holstein	Emilia Romagna	34.7	951	951	951
2	Holstein	Emilia Romagna	34.3	72	72	72
3	Holstein	Veneto	30.2	129	129	129
4	Holstein	Veneto	33.2	39	39	39
5	Holstein	Veneto	33.7	13	13	13
6 to 33 ³	Multibreed	Trentino Alto Adige	28.8	—	1,497	1,507
Total (N = 33)	—	—	—	1,204	2,701	2,711

¹The complete blood profile comprised all the blood metabolites considered in this study except for BHB.

²Fourier transform mid-infrared (FTIR) spectral data available for this study.

³All the herds sampled in Trentino Alto Adige were grouped together because there were no differences among them in providing phenotypic and FTIR spectra information.

This sample of cows included dairy cows at all stages of lactation (DIM range, 5 to >305), and with parities ranging from 1 to 7. The cows were housed mostly in sand-bedded free stalls and fed twice daily on a TMR based on corn and sorghum silage or hay (multibreed farms of the Trentino Alto Adige region) supplemented with concentrates. All lactating cows received the same TMR as part of routine herd management. Drinking water was available from automatic water bowls. The cows were milked twice a day (morning and evening, with an interval of 12 h between sessions) in a double-12 herringbone milking parlor. Cows with clinical signs of disease (e.g., retained placenta, metritis, clinical mastitis, abomasal displacement, uterine prolapse) or that had received medical treatment were not sampled.

Milk Sampling

Milk samples were collected once during the evening milking from individual cows in 61 batches (i.e., herd/date combinations): 41 batches in 2013 (1,521 cows), 16 in 2019 (1,032 cows), and 4 in 2020 (188 cows). Large herds were sampled in more than 1 d because the laboratory could only process ~65 milk samples/d. After collection, the individual milk samples were kept at 4°C until analysis. A preservative (bronopol) was added to each milk sample, which was transferred to the laboratory of the Breeders Association of the Veneto or of the Province of Trento (Trento, Italy) for milk quality and composition analyses, and FTIR spectra acquisition.

Blood Sampling, Hematochemical Analysis, and Data Preprocessing

Blood samples (5 mL) were collected from the jugular vein in the morning before TMR distribution and placed in vacuum tubes containing 150 lithium heparin USP units (Vacumed, FL Medical). Blood and milk

sampling of each cow were carried out concurrently or proximately.

A complete blood metabolic profile comprising 29 blood metabolites was obtained from 1,209 Holstein-Friesian cows belonging to 5 herds in accordance with the protocols described by Mezzetti et al. (2019) (Table 1). Briefly, blood samples were kept on ice until centrifugation at $3,500 \times g$ for 16 min at 6°C (Hettich Universal 16R Centrifuge), which was performed within 2 h of collection. The plasma thus obtained was stored at -20°C until further analysis. A small fraction of blood was used to determine the hematocrit (ALC Centrifuge 4203; $15,300 \times g$, 12 min at room temperature). An ILAB-650 clinical autoanalyzer (Instrumentation Laboratory, Werfen) was used to determine the concentrations of glucose, NEFA, BHB, urea, creatinine, Ca, P, Mg, Na, K, Cl, Zn, aspartate aminotransferase (**AST**), γ -glutamyl transferase (**GGT**), alkaline phosphatase (**ALP**), total proteins, haptoglobin, ceruloplasmin, albumin, total bilirubin (**BILt**), cholesterol, total reactive oxygen metabolites (**ROMt**), advanced oxidation protein products (**AOPP**), ferric reducing antioxidant power (**FRAP**), total thiol groups of plasma (**SHp**), paraoxonase, and myeloperoxidase. Globulin concentration was calculated as total proteins concentration minus albumin concentration.

For the 1,521 cows pertaining to the Cowplus project, BHB blood levels were determined with a Cobas C-501 analyzer (Roche Diagnostics), as described in Cecchinato et al. (2018).

We tested various mathematical treatments of the blood metabolite data before model development, but because no improvement was observed using transformed values for prediction, we analyzed the original variables. The frequency distributions of all blood metabolites are depicted in Supplemental Figures S1 to S5 (<https://doi.org/10.6084/m9.figshare.21739655>; Giannuzzi, 2022).

Milk FTIR Spectra Analysis and Preprocessing

Milk FTIR spectra were acquired and analyzed with a MilkoScan FT6000 (Foss A/S). They consisted of the transmittance values measured at 1,060 wavenumbers, ranging from 5,011 to 925 cm^{-1} . The spectra were expressed as an absorbance value $[\log(1/\text{transmittance})]$. Principal component analysis of the FTIR spectral data was performed and the Mahalanobis distance calculated at a significance level of 5% to remove possible outliers. After this data-cleaning step, 2,711 cows remained for the analysis: 1,507 sampled in 2013, 1,024 in 2019, and 180 in 2020.

Cross-Validation Scenarios

Fourier-transform mid-infrared predictions of the blood metabolites were assessed with 3 CV scenarios—namely 5-fold random (**CV_r**), herd-out (**CV_h**), and true-positive prediction (**TPP**), with individuals assigned to the training and validation populations completely independently of each other. The model fit of the parameters was assessed by coefficient of determination (R^2), root mean square error (**RMSE**), and the slope of linear regression between the observed and predicted phenotypes.

Random 5-Fold. To assess the predictive ability, the data set was split randomly into 5 portions of equal size, with 4 used for training and 1 used for validation. This process was repeated 5 times, and the average was calculated and used to assess predictive ability.

Herd-Out. The **CV_h** scenario was carried out to provide a full block-cross-validation. In this scenario, 5 herds (herds 1–5) were present. One of them (herd 1, with 951 cows) was maintained as the fixed training population, and the others, in turn, were used as validation set. Thus, the process was repeated 4 times for herds 2, 3, 4, and 5. For BHB, which had 2,701 cows and 33 herds, the **CV_h** scenario was built with 1 herd assigned as a validation set and 32 herds as the training set. The process was repeated 33 times for each herd to be predicted in the validation set.

True-Positive Prediction. This specific scenario aimed at evaluating the predicting ability of milk FTIR spectra within the tails of the population. This appears particularly useful for blood metabolites without a defined risk threshold during metabolic disturbances, such as oxidative stress metabolites or inflammation-related parameters. Indeed, individuals with values in the tails could be considered at risk of developing subclinical or clinical disease. First, each hematochemical parameter was discretized and 3 classes were created on the basis of Q25 and Q75. The data set was then split into the 3 classes, and 80% of each class was assigned randomly

to the training set, with the remaining 20% assigned to the validation set. The process was replicated 5 times and the TPP within each class was evaluated. The TPP for the 2 extreme classes (i.e., Q25 and Q75) was calculated as the percentage of all predictions of a given class that were correct.

Statistical Analysis

The target phenotype (i.e., the blood metabolite) at the i th individual (y_i) was predicted given the FTIR spectrum wavelength (x_{ij}), with j ranging from 1 to 1,060. The automatic machine learning (**autoML**) function of the **h2o** R package (R Foundation for Statistical Computing, <https://github.com/h2oai/h2o-3>), which trains and adjusts the models automatically, was used. To find the model with the best prediction accuracy, **autoML** was run using 4 ML models [distributed random forest (**DRF**), gradient boosting machine (**GBM**), XGBoost (**XGB**), and multilayer feed-forward artificial neural network (**ANN**)], 3 penalized regression models [ridge regression (**RR**), least absolute shrinkage and selection operator (**LASSO**), and elastic net (**EN**)], and stacking ensemble, which uses a combination of the best predictions achieved by the previously cited models. The default settings of the **autoML** software in the **h2o** R package were used, with the options `max_models = 200` and `max_runtime_secs = 9,000`.

Penalized Regressions. These type of models perform phenotypic prediction using a single penalization $\lambda_1 = \sum \beta_j^2$ (**RR**) or $\lambda_2 = \sum |\beta_j|$ (**LASSO**), or a combination of **RR** and **LASSO** penalties (Zou and Hastie, 2005). In the **EN** model, a combination of **RR** (λ_1) and **LASSO** (λ_2) penalties controls the strength between the selection (**LASSO**) and the shrinkage (**RR**) of the predictor variables. The penalized regression models are thus able to minimize the loss function (L , in the following calculation) according to the values of λ_1 and λ_2 as follows:

$$L(\lambda_1, \lambda_2, \beta) = \min \left\{ \frac{1}{2N} \sum_{i=1}^N \left[y_i - \left(\beta_0 + \sum_{j=1}^{1,060} x_{ij} \beta_j \right) \right]^2 + (1 - \alpha) |\beta_j| + \alpha \beta_j^2 \right\},$$

where N is the number of animals for each trait, β_0 is the intercept of the regression, β_j is the regression coefficient value, y_i is the target phenotype at the i th individual, and x_{ij} is the FTIR infrared value. The penalized regression simplifies to **RR** when $\alpha = 1$, and to **LASSO** when $\alpha = 0$. The λ_1 part of the **EN** does automatic variable selection, whereas the λ_2 part encourages

grouped selection and stabilizes the solution paths with respect to random sampling, which improves phenotype prediction. In this case, a random search was performed to find the optimal value of λ with `lambda_search` enabled and α with values of 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0. During the training processes, EN always achieved better predictive ability against RR and LASSO regression; therefore, in the tables, only the predictive ability of EN is described.

Distributed Random Forest, GBM, and XGBoost. These are regression tree models that combine individual trees through bootstrap aggregation (DRF) or boosting (GBM and XGBoost). The DRF algorithm fits different regression trees by dividing the training population into different subsets, and then averaging them into the final predictive model. The GBM and XGBoost approaches combine gradient descent error minimization with boosting to convert weak learners (i.e., regression trees) into strong learners using the algorithm specified by Hastie et al. (2009). To implement the GBM, a random search of hyperparameters was performed with the number of trees (Ntree) set at 10,000 default within the autoML function; the learning rate, which controls the size of the steps in the gradient descent, was set at 0.1; the maximum tree depth was set at 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, and 17; the minimum sample per leaf was set at 1, 5, 10, 15, 30, and 100; column sampling and column sampling per tree had values of 0.4, 0.7, and 1; and the sampling rate was set at 0.5, 0.6, 0.7, 0.8, 0.9, and 1. In addition, the XGBoost also considers the α values (0.001, 0.01, 0.1, 1, 10, and 100) and λ regularization values (0.001, 0.01, 0.1, 0.5, and 1). In DRF, the Ntree was set at 10,000, and the mtries was $\sqrt{M}$, where M is the number of FTIR predictor variables (Briec et al., 2018).

Multilayer Feed-Forward ANN. The ANN method processes nonlinear transformations of the predictor variables through multiple hidden layers before making the phenotypic prediction on the output layer. The number of hidden layers determines the depth of the neural network, whereas the number of neurons in each hidden layer determines its width. The ANN is trained through an iterative process using feed-forward, which calculates the weights from the hidden layers to the output layer, whereas backpropagation passes information from the loss function to update the weights used in the model (Rumelhart et al., 1986) with the aim of reducing the mean square error (the loss function). The ANN hyperparameters selected via random search and tuned were the activation function (RectifierWithDropout); epochs (10,000); hidden layer and number of neurons grid search 1, (20), (50), and (100); grid search

2, (20, 20), (50, 50), and (100, 100); and grid search 3, (20, 20, 20), (50, 50, 50), and (100, 100, 100); hidden dropout ratio grid search 1, (0.1), (0.2), (0.3), (0.4), and (0.5); grid search 2, (0.1, 0.1), (0.2, 0.2), (0.3, 0.3), (0.4, 0.4), and (0.5, 0.5); and grid search 3, (0.1, 0.1, 0.1), (0.2, 0.2, 0.2), (0.3, 0.3, 0.3), (0.4, 0.4, 0.4), and (0.5, 0.5, 0.5); input dropout ratio, 0.0, 0.05, 0.1, 0.15, and 0.2; and ρ , 0.9, 0.95, and 0.99.

Stacking Ensemble. The stacking ensemble uses a meta-learning algorithm to combine the models generated by the different ML algorithms and trained previously to make a final prediction (Wolpert, 1992; Breiman, 1996). The stacking ensemble prediction was performed in 2 steps. In the first, output predictions are obtained from the base learner (DRF, GBM, ANN, XGBoost, and EN) trained previously with the autoML. In the second step, the final prediction is obtained through a generalized linear model with a linear combination of weights ($a_i, \dots, a_n$) as follows:

$$f_{\text{stacking}} = \sum_{i=1}^n a_i f_i(x),$$

where $f_i(x)$ represents the phenotype predicted from each base learner and a_i is the weight vector learned in the meta-learner.

Partial Least Squares Regression. To compare the models' performance with a traditionally used prediction method for blood traits, equations for blood metabolic profile prediction were also developed using PLS regression. The PLS regression was fitted using the pls package in R software [R Foundation for Statistical Computing (Mevik and Wehrens, 2007)]. The number of latent variables included in each calibration equation was determined based on the model having the smallest RMSE, maximizing the percentage of variance captured for each trait. The model performance was evaluated with the 2 CV scenarios of CV_r and the CV_h , corresponding to those set up for the autoML tool.

RESULTS

Blood Metabolic Profile of Cows

Descriptive statistics of the blood metabolite concentrations in the investigated cohort are reported in Supplemental Table S1 (<https://doi.org/10.6084/m9.figshare.21739655>; Giannuzzi, 2022). Examining all the BHB records ($n = 2,701$), we found that BHB levels were high (>1 mmol/L; Ospina et al., 2010) in less than 3% ($n = 77$) of cows. Considering the cows for which we were able to evaluate the entire blood metabolic profile ($n = 1,204$), 2% showed serum BHB levels greater than

1 mmol/L, whereas none of those cows had high NEFA concentrations (≥ 0.7 mmol/L; Ospina et al., 2010). We observed a certain degree of serum protein alteration. Sixteen percent of cows had elevated globulin concentrations (>50 g/L), whereas only 1% had very low albumin concentrations (<30 g/L; Whitaker, 2004). A relevant proportion of cows (24%) showed increased blood levels of haptoglobin (>0.30 g/L), and 14% had serum levels greater than 0.6 g/L (Martins et al., 2021; Premi et al., 2021). Ceruloplasmin was increased (>3.2 mmol/L) in 6% of cows (Premi et al., 2021). The urea concentration threshold of ≥ 6.78 mmol/L (Butler et al., 1996) was exceeded in 31% of cows, and none had serum urea concentrations less than the optimal range (<1.7 mmol/L; Macrae et al., 2006). Approximately 3% of cows had elevated BILt (>4 mmol/L; Giannuzzi et al., 2021), whereas less than 1% were suspected of having hypomagnesemia ($\text{Mg} < 0.7$ mmol/L) or hypocalcemia ($\text{Ca} < 2.0$ mmol/L; Martín-Tereso and Martens, 2014).

Prediction Model Performances for Random and Herd-Out CV Scenarios

Regarding the different ML methods applied by the autoML tool, the stacking ensemble achieved the highest prediction ability (R^2) for the majority of the blood metabolites across the 2 CV scenarios. Exceptions were seen in total proteins and Cl, for which the best method was EN for both CV scenarios; hematocrit, creatinine, albumin, and FRAP, for which the best method was EN only for CV_r ; and urea, BILt, ROMt, and Na, for which the best method was EN only for CV_h . Overall, the stacking ensemble performed better than the DRF, GBM, XGB, and ANN base learners, and was more similar to EN. The details of fitting statistics of the PLS and the other ML algorithms (CV_r and CV_h scenarios), for the different categories of blood metabolites, are shown in Tables 2, 3, 4, 5, and 6.

To assess the performance of the ML models against a conventional method for blood trait prediction using milk FTIR spectra, we compared the prediction accuracies of the CV_r and CV_h scenarios with PLS regression. The best-performing algorithms selected by autoML (EN and stacking ensemble) showed an increase in prediction ability over PLS regression. More precisely, the prediction accuracy using EN increased from 5% to 75% for CV_r and 2% to 139% for CV_h , whereas the stacking ensemble increased the R^2 from 4% to 70% for CV_r and 4% to 150% for CV_h against PLS regression.

The CV_h scenario showed more conservative results, with a lower predictive ability than the CV_r scenario, with the highest reduction in R^2 exhibited by haptoglobin (−60%), FRAP (−48%), and urea (−34%).

Table 2. Average model predictive performance (R^2) and root mean square error (RMSE) of the diverse machine learning algorithms for hematocrit and energy-related metabolites across random cross-validation (CV_r) and herd-out cross-validation (CV_h) scenarios

Blood metabolite ¹	CV	PLS ²		DRF ²		XGB ²		Elastic net		GBM ²		ANN ²		Stacking ²	
		R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE
Hematocrit, L/L	CV_r	0.08	0.03	0.11	0.03	0.11	0.03	0.14	0.03	0.13	0.03	0.09	0.03	0.13	0.03
	CV_h	0.08	0.03	0.09	0.03	0.08	0.03	0.12	0.03	0.10	0.03	0.09	0.06	0.15	0.03
Energy-related metabolite	CV_r	0.71	0.19	0.66	0.25	0.72	0.20	0.80	0.17	0.75	0.20	0.76	0.19	0.81	0.17
	CV_h	0.60	0.32	0.55	0.36	0.58	0.31	0.70	0.30	0.69	0.30	0.65	0.37	0.70	0.30
Cholesterol, mmol/L	CV_r	0.44	0.97	0.42	0.95	0.5	0.88	0.55	0.84	0.49	0.89	0.52	0.87	0.57	0.81
	CV_h	0.39	1.38	0.36	1.05	0.35	1.02	0.55	1.08	0.42	0.93	0.52	0.98	0.58	0.91
NEFA, mmol/L	CV_r	0.60	0.06	0.52	0.06	0.57	0.06	0.65	0.05	0.61	0.06	0.49	0.07	0.68	0.05
	CV_h	0.46	0.11	0.62	0.09	0.71	0.08	0.70	0.08	0.64	0.09	0.64	0.11	0.78	0.08
BHB, mmol/L	CV_r	0.39	0.14	0.41	0.15	0.48	0.13	0.57	0.12	0.47	0.14	0.54	0.13	0.61	0.12
	CV_h	0.18	0.17	0.26	0.16	0.31	0.14	0.43	0.14	0.31	0.14	0.39	0.14	0.45	0.13
Urea, mmol/L	CV_r	0.59	0.68	0.52	0.79	0.61	0.71	0.71	0.61	0.63	0.69	0.69	0.63	0.73	0.60
	CV_h	0.31	1.01	0.33	1.09	0.34	1.04	0.49	1.17	0.33	0.97	0.36	1.10	0.48	1.10
Creatinine, $\mu\text{mol/L}$	CV_r	0.48	4.88	0.47	5.19	0.48	4.97	0.59	4.33	0.51	4.78	0.50	5.61	0.59	4.38
	CV_h	0.45	6.66	0.38	6.55	0.54	5.84	0.6	7.43	0.54	5.78	0.57	7.78	0.60	6.96

¹NEFA = nonesterified fatty acids.

²PLS = partial least squares regression; DRF = distributed random forest; XGB = xgboost; GBM = gradient boosting machine; ANN = feedforward artificial neural network; stacking = stacking ensemble.

Table 3. Average model predictive performance (R^2) and root mean square error (RMSE) of the diverse machine learning algorithms for blood metabolites related to liver function and hepatic damage across random cross-validation (CV_r) and herd-out cross-validation (CV_h) scenarios

Blood metabolite ¹	CV	PLS ²		DRF ²		XGB ²		Elastic net		GBM ²		ANN ²		Stacking ²	
		R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE
Liver function/hepatic damage	AST, U/L	CV_r	0.50	16.38	0.47	16.81	0.51	15.84	0.57	14.89	0.56	15.12	0.49	16.51	0.62
		CV_h	0.31	21.96	0.46	21.14	0.51	17.77	0.50	17.99	0.51	19.01	0.52	19.95	0.58
	GGT, U/L	CV_r	0.45	5.7	0.40	5.81	0.40	5.80	0.55	5.04	0.48	5.38	0.32	6.47	0.55
		CV_h	0.39	6.59	0.33	6.81	0.30	6.87	0.56	5.26	0.35	6.54	0.32	6.47	0.55
Albumin, g/L	BILt, μ mol/L	CV_r	0.60	0.55	0.56	0.56	0.54	0.57	0.68	0.49	0.61	0.52	0.53	0.59	0.68
		CV_h	0.46	0.70	0.41	0.63	0.49	0.61	0.66	0.59	0.44	0.61	0.56	0.59	0.62
	Albumin, g/L	CV_r	0.62	1.16	0.61	1.37	0.66	1.27	0.75	1.07	0.65	1.29	0.65	1.3	0.75
		CV_h	0.55	1.57	0.44	1.85	0.37	1.87	0.64	1.32	0.45	1.70	0.63	1.35	0.65
ALP, U/L	ALP, U/L	CV_r	0.57	13.08	0.45	14.39	0.52	13.48	0.62	11.97	0.61	12.22	0.44	14.85	0.65
		CV_h	0.30	17.19	0.19	16.35	0.37	13.81	0.54	13.7	0.34	14.03	0.56	13.96	0.54
	PON, U/mL	CV_r	0.48	14.1	0.41	14.79	0.40	14.75	0.55	12.75	0.50	13.52	0.36	15.41	0.58
		CV_h	0.30	18.3	0.24	17.94	0.41	15.13	0.42	16.6	0.48	14.47	0.48	13.64	0.46

¹AST = aspartate aminotransferase; GGT = γ -glutamyl transferase; BILt = total bilirubin; ALP = alkaline phosphatase; PON = paraoxonase.²PLS = partial least squares regression; DRF = distributed random forest; XGB = xgboost; GBM = gradient boosting machine; ANN = feedforward artificial neural network; stacking = stacking ensemble.**Table 4.** Average model predictive performance (R^2) and root mean square error (RMSE) of the diverse machine learning algorithms for oxidative stress metabolites across random cross-validation (CV_r) and herd-out cross-validation (CV_h) scenarios

Blood metabolite ¹	CV	PLS ²		DRF ²		XGB ²		Elastic net		GBM ²		ANN ²		Stacking ²	
		R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE
Oxidative stress metabolite	ROMt, mg of H ₂ O ₂ /100 mL	CV_r	0.60	2.04	0.71	2.03	0.74	1.92	0.78	1.77	0.74	1.94	0.75	1.87	0.79
		CV_h	0.52	2.52	0.55	2.88	0.56	2.61	0.66	2.06	0.53	2.61	0.42	2.97	0.61
	AOPP, μ mol/L	CV_r	0.55	6.77	0.53	6.73	0.51	6.81	0.63	5.95	0.58	6.32	0.42	7.55	0.65
		CV_h	0.51	14.70	0.47	14.64	0.45	13.69	0.60	13.32	0.51	14.07	0.38	13.58	0.61
FRAP, μ mol/L	FRAP, μ mol/L	CV_r	0.33	26.45	0.35	30.56	0.42	28.89	0.57	25.39	0.47	27.56	0.28	32.62	0.56
		CV_h	0.15	43.25	0.18	39.21	0.21	38.03	0.28	41.49	0.21	33.42	0.15	39.29	0.29
	SHp, μ mol/L	CV_r	0.62	33.86	0.62	37.48	0.67	34.84	0.75	31.03	0.70	33.21	0.62	37.60	0.76
		CV_h	0.44	56.58	0.31	60.83	0.38	57.33	0.61	48.34	0.39	55.62	0.45	57.01	0.61

¹ROMt = total reactive oxygen metabolites; AOPP = advanced oxidation protein products; FRAP = ferric reducing antioxidant power; SHp = total thiol groups.²PLS = partial least squares regression; DRF = distributed random forest; XGB = xgboost; GBM = gradient boosting machine; ANN = feedforward artificial neural network; stacking = stacking ensemble.

Table 5. Average model predictive performance (R^2) and root mean square error (RMSE) of the diverse machine learning algorithms for blood metabolites related to inflammation and innate immunity in random cross-validation (CV_r) and herd-out cross-validation (CV_h) scenarios

Blood metabolite ¹	CV	PLS ²		DRF ²		XGB ²		Elastic net		GBM ²		ANN ²		Stacking ²	
		R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE
Inflammation/innate immunity															
Ceruloplasmin, $\mu\text{mol/L}$	CV_r	0.69	0.38	0.65	0.40	0.69	0.38	0.73	0.35	0.69	0.38	0.69	0.38	0.74	0.34
	CV_h	0.46	0.56	0.48	0.55	0.45	0.58	0.60	0.46	0.53	0.57	0.41	0.67	0.60	0.47
PROTt, g/L	CV_r	0.78	2.49	0.73	2.80	0.76	2.61	0.82	2.23	0.77	2.56	0.73	2.75	0.81	2.44
	CV_h	0.7	3.87	0.66	3.49	0.64	3.30	0.75	2.85	0.68	3.23	0.65	3.50	0.75	2.96
Globulins, g/L	CV_r	0.83	2.43	0.79	2.70	0.8	2.56	0.87	2.12	0.83	2.40	0.81	2.55	0.87	2.07
	CV_h	0.82	3.19	0.62	3.71	0.78	2.88	0.84	2.41	0.72	3.16	0.81	2.72	0.85	2.40
Haptoglobin, g/L	CV_r	0.34	0.20	0.35	0.20	0.4	0.19	0.43	0.18	0.39	0.19	0.44	0.19	0.48	0.18
	CV_h	0.12	0.22	0.14	0.21	0.10	0.21	0.16	0.21	0.12	0.20	0.18	0.24	0.21	0.19
Myeloperoxidase, U/L	CV_r	0.57	52.55	0.53	54.10	0.57	51.70	0.62	49.52	0.6	49.49	0.46	58.81	0.64	47.07
	CV_h	0.23	106.37	0.28	82.49	0.32	80.23	0.46	82.99	0.42	75.69	0.48	80.59	0.51	76.33

¹PROTt = total proteins.²PLS = partial least squares regression; DRF = distributed random forest; XGB = xgboost; GBM = gradient boosting machine; ANN = feedforward artificial neural network; stacking = stacking ensemble.**Table 6.** Average model predictive performance (R^2) and root mean square error (RMSE) of the diverse machine learning algorithms for blood minerals in random cross-validation (CV_r) and herd-out cross-validation (CV_h) scenarios

Blood metabolite	CV	PLS ¹		DRF ¹		XGB ¹		Elastic net		GBM ¹		ANN ¹		Stacking ¹	
		R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE
Mineral															
Calcium, mmol/L	CV_r	0.45	0.09	0.48	0.08	0.52	0.08	0.56	0.08	0.51	0.08	0.52	0.08	0.57	0.08
	CV_h	0.20	0.14	0.55	0.10	0.54	0.10	0.62	0.10	0.52	0.10	0.56	0.13	0.63	0.11
Phosphorus, mmol/L	CV_r	0.63	0.19	0.53	0.21	0.59	0.19	0.68	0.17	0.64	0.18	0.66	0.18	0.70	0.17
	CV_h	0.35	0.27	0.37	0.25	0.51	0.23	0.65	0.20	0.58	0.21	0.48	0.24	0.66	0.19
Magnesium, mmol/L	CV_r	0.51	0.07	0.45	0.07	0.49	0.07	0.57	0.06	0.51	0.06	0.54	0.06	0.58	0.06
	CV_h	0.33	0.08	0.26	0.08	0.26	0.09	0.46	0.08	0.29	0.08	0.34	0.08	0.46	0.08
Sodium, mmol/L	CV_r	0.65	1.69	0.63	1.73	0.66	1.62	0.71	1.51	0.67	1.61	0.68	1.61	0.72	1.49
	CV_h	0.45	2.17	0.53	2.07	0.50	2.18	0.68	2.13	0.59	2.04	0.61	2.33	0.68	3.01
Potassium, mmol/L	CV_r	0.49	0.29	0.50	0.28	0.55	0.27	0.59	0.26	0.56	0.26	0.53	0.28	0.60	0.25
	CV_h	0.27	0.50	0.36	0.37	0.41	0.36	0.51	0.49	0.43	0.35	0.48	0.50	0.52	0.46
Chlorine, mmol/L	CV_r	0.65	1.74	0.59	1.89	0.63	1.79	0.71	1.58	0.65	1.75	0.66	1.72	0.71	1.74
	CV_h	0.49	2.47	0.38	2.75	0.47	2.62	0.58	2.30	0.45	2.61	0.34	2.94	0.58	3.21
Zinc, $\mu\text{mol/L}$	CV_r	0.51	1.95	0.42	2.06	0.46	1.98	0.58	1.76	0.47	1.97	0.53	1.86	0.58	1.75
	CV_h	0.34	3.43	0.44	2.05	0.47	1.98	0.58	1.76	0.49	1.96	0.55	1.84	0.59	1.74

¹PLS = partial least squares regression; DRF = distributed random forest; XGB = xgboost; GBM = gradient boosting machine; ANN = feedforward artificial neural network; stacking = stacking ensemble.

However, the stacking ensemble exhibited a greater adaptability to learning during training, with a lower reduction (−2% to −56%) in R^2 compared with PLS (−30% to −65%), DRF (−2% to −60%), XGB (−3% to −75%), EN (−3% to −63%), GBM (−4% to −69%), and ANN (−2% to −59%). Exceptions were seen in NEFA and Ca, where we observed an increase in R^2 of 15% (Table 2) and 11% (Table 6), respectively, in the CV_h compared with the CV_r scenarios. Also, the GGT prediction showed a greater increment of R^2 (+60%) in CV_h compared with CV_r when using the ANN approach (Table 3). Model fit assessed by RMSE revealed that the residual parameters of the CV_r scenario were less than those of CV_h , with the average decrease being 26.3% for all parameters (ranging from 0% of hematocrit to 57% of AOPP). Consistent with this, the estimate of inflation using the slope coefficient, which showed the prediction bias, was the same for both CV scenarios, although the bias was slightly less for most of the parameters when assessed with CV_r (data not shown).

From this point forward, R^2 of the best-performing algorithm for each metabolite was used for comparison between CV scenarios and subsequent analyses (TPP and variable importance assessment). On average, the metabolites belonging to the inflammation and innate immunity biological category had the greatest predictive ability (average $R^2 = 0.71$ for CV_r), whereas blood minerals had the least (average $R^2 = 0.64$ for CV_r). More specifically, the greatest predictive ability was achieved by globulins for both CV scenarios ($R^2 = 0.87$ for CV_r and $R^2 = 0.85$ for CV_h ; Table 6), and the least by hematocrit ($R^2 = 0.14$ for CV_r , $R^2 = 0.15$ for CV_h ; Table 3) and haptoglobin ($R^2 = 0.48$ for CV_r , $R^2 = 0.21$ for CV_h ; Table 6). The most promising results for serum concentration predictions of energy-related metabolites were obtained for glucose ($R^2 = 0.81$ for CV_r , $R^2 = 0.70$ for CV_h), NEFA ($R^2 = 0.68$ for CV_r , $R^2 = 0.78$ for CV_h), and urea ($R^2 = 0.73$ for CV_r , $R^2 = 0.49$ for CV_h ; Table 2). With regard to liver function and hepatic damage metabolites, accurate predictive ability was found for serum concentrations of BILt ($R^2 = 0.68$ for CV_r , $R^2 = 0.66$ for CV_h) and albumin ($R^2 = 0.75$ for CV_r , $R^2 = 0.65$ for CV_h ; Table 3). Among the oxidative stress metabolites, serum concentrations exhibited good prediction accuracies for ROMt ($R^2 = 0.79$ for CV_r , $R^2 = 0.66$ for CV_h) and SHp ($R^2 = 0.76$ for CV_r , $R^2 = 0.61$ for CV_h ; Table 4). Of the inflammation-related metabolites, apart from globulins, prediction accuracy was also good for ceruloplasmin ($R^2 = 0.74$ for CV_r , $R^2 = 0.60$ for CV_h) and total proteins ($R^2 = 0.81$ for CV_r , $R^2 = 0.75$ for CV_h ; Table 5). Last, of the serum minerals, the most accurate predictions were for serum concentrations of Na ($R^2 = 0.72$ for CV_r , $R^2 = 0.68$ for

CV_h) and Cl ($R^2 = 0.71$ for CV_r , $R^2 = 0.58$ for CV_h ; Table 6).

Precision of Classification Using the TPP CV Scenario

Figure 1 shows the TPP (precision of classification, %) obtained by the prediction equations for predicted values in the extreme classes Q25 and Q75. Among the energy-related blood metabolites, we obtained a good TPP (>70%) in classifying predicted values of glucose in Q25 (70.8%) and Q75 (69.9%; Figure 1a). Classification of NEFA and urea in Q75 was satisfactory (65 and 66.7%, respectively; Figure 1a). For the other blood metabolites, TPP in the extreme classes was unsatisfactory, particularly for NEFA and BHB in Q25 (<35%; Figure 1a).

Regarding liver function blood parameters, predicted values for albumin were classified accurately in Q25 (72.3%; Figure 1b). The TPP was satisfactory for AST, BILt, albumin, and ALP in Q75 (67.4, 68.4, 68.5, and 68.2%, respectively; Figure 1b). Among the oxidative stress metabolites, the TPP was good in the case of ROMt in both Q25 and Q75 (75.1 and 74%, respectively), and in the case of SHp in Q75 (70.4%; Figure 1c). Advanced oxidation protein products and SHp predicted values were classified with satisfactory precision in the Q25 class (62 and 61.1%, respectively; Figure 1c).

Among the indicators of inflammation, we obtained a good TPP in classifying predicted values of total proteins (72.4%) and globulins (74.8%) in Q25, and of total proteins (77.2%), globulins (81.5%), and haptoglobin (74.4%) in Q75 (Figure 1d). Classification in both Q25 and Q75 was satisfactory for ceruloplasmin (67.7 and 64.9%, respectively) and myeloperoxidase (62.2 and 64.9%, respectively; Figure 1d). In contrast, haptoglobin predicted values in Q25 were completely misclassified, with a very poor TPP value (<20%; Figure 1d).

Last, blood mineral predicted values were all classified unsatisfactorily in the 2 extreme classes, except for P and Cl predicted values in Q25 (67.9 and 69.2%, respectively) and Na in Q75 (69.3%; Figure 1e).

Relevance of FTIR Wavelengths in Explaining the Variation in Blood Traits

The whole blood metabolic profile was captured from precise FTIR wavelength regions, which were also consistent with the biological category to which each hematochemical parameter belonged (Figures 2 to 6). Overall, the wavelengths that made the greatest contribution (>0.2% of explained importance) to

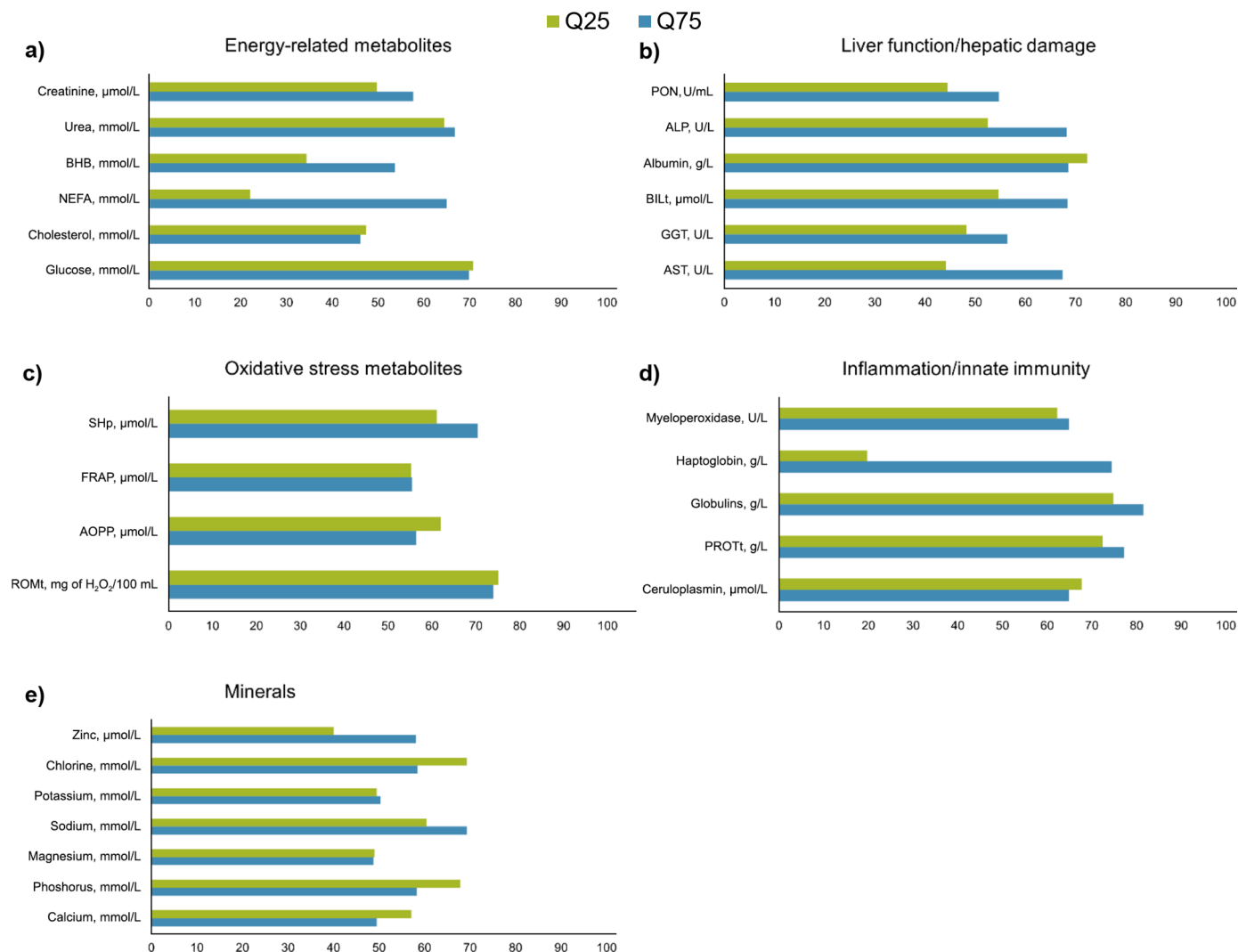


Figure 1. Cross-validation with true-positive prediction scenario. Precision of classification (%) obtained by the prediction equations to classifying predicted values correctly in the extreme classes of the 25th and 75th percentiles (Q25 and Q75, respectively) for the 5 categories of blood metabolites. NEFA = nonesterified fatty acids; PON = paraoxonase; ALP = alkaline phosphatase; BILt = total bilirubin; GGT = γ -glutamyl transferase; AST = aspartate aminotransferase; SHp = total thiol groups; FRAP = ferric reducing antioxidant power; AOPP = advanced oxidation protein products; ROMt = total reactive oxygen metabolites; PROTt = total proteins.

achieving the best prediction covered 3 main regions of the FTIR spectrum: the short-wavelength infrared (**SWIR**) and mid-wavelength infrared (**MWIR**; 3,669 to 3,052 cm^{-1}), the MWIR2 (1,698 to 1,586 cm^{-1}), and the MWIR and long-wavelength infrared (**LWIR**; 1,584 to 925 cm^{-1} ; Bittante and Cecchinato, 2013).

For energy-related blood metabolites, wavelengths in the extreme portion of the LWIR, from 929 to 971 cm^{-1} , are particularly relevant (Figure 2). The value of 971 cm^{-1} was shared by all the energy-related blood traits: 956 cm^{-1} , by all the traits except BHB; 940 cm^{-1} , by glucose, cholesterol, creatinine, and urea; and 964 cm^{-1} , by glucose, BHB, and creatinine. Of note, glucose and urea also had relevant contributions from wavelengths

in the SWIR-MWIR transition region between 3,708 and 3,650 cm^{-1} , with the greatest contribution for glucose prediction pertaining to wavelength 4,351 cm^{-1} (8.3%). Altogether, these regions contributed 0.5% to 8.3% to the predictive ability of the algorithm.

Regarding the more informative wavelengths for the prediction of hepatic function-related metabolites, the region located between 975 and 925 cm^{-1} made the greatest contribution (Figure 3). A wavelength of 924 cm^{-1} was shared by ALP, BILt, and GGT, with the latter making a contribution of 11.6%. A wavelength of 956 cm^{-1} was shared by ALP, BILt, albumin, and AST, and explained 13% of the model's predictive ability for those variables. In addition, the region between

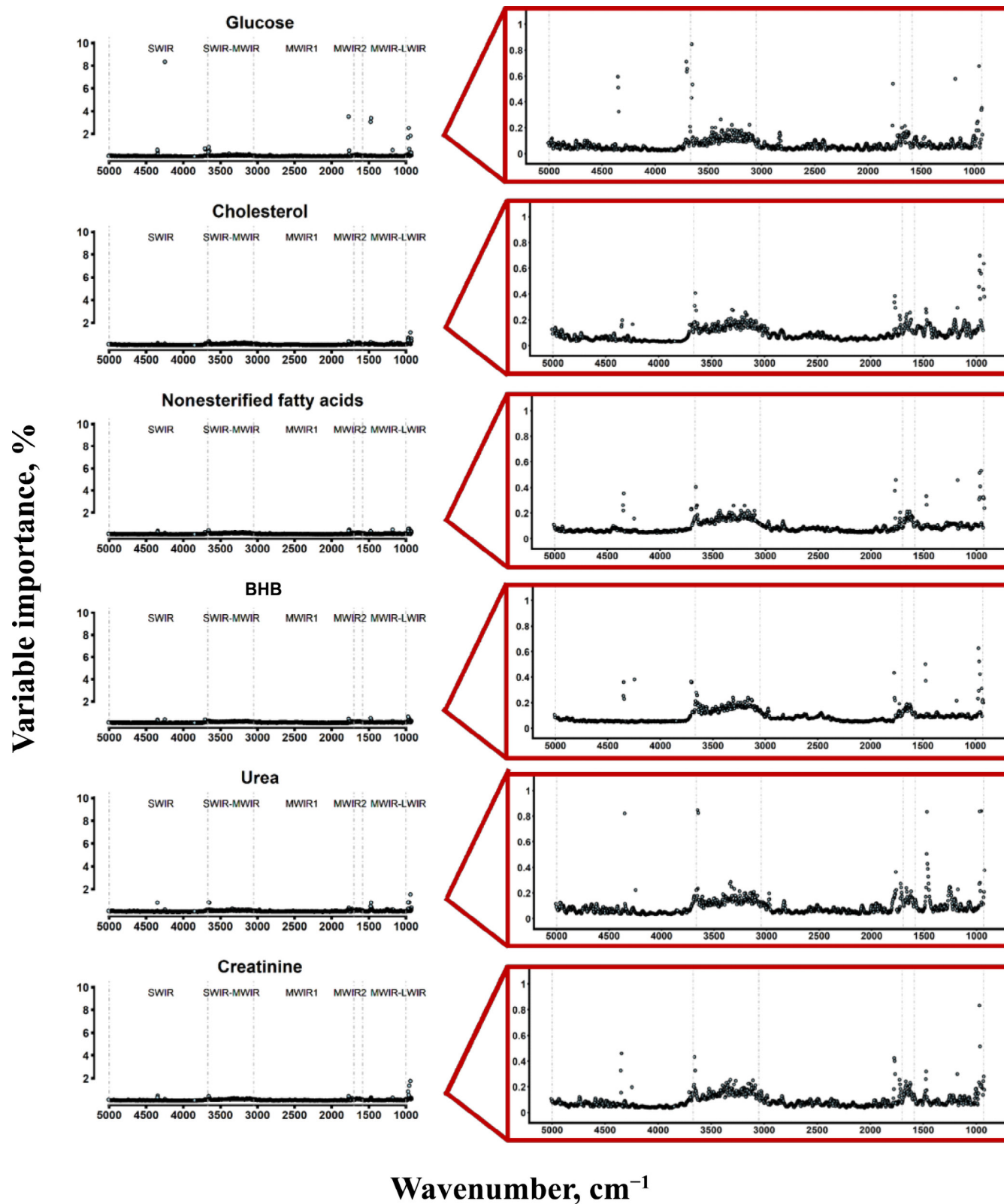


Figure 2. Variable importance values for the best prediction model selected by the automatic machine learning tool across the entire Fourier-transform mid-infrared spectrum (1,060 wavelengths) for energy-related blood metabolites. In the red square is a magnification of the lowest part of the y -axis (<1.5%) to appreciate each wavelength's contribution. SWIR = short-wavelength infrared; MWIR = mid-wavelength infrared; LWIR = long-wavelength infrared.

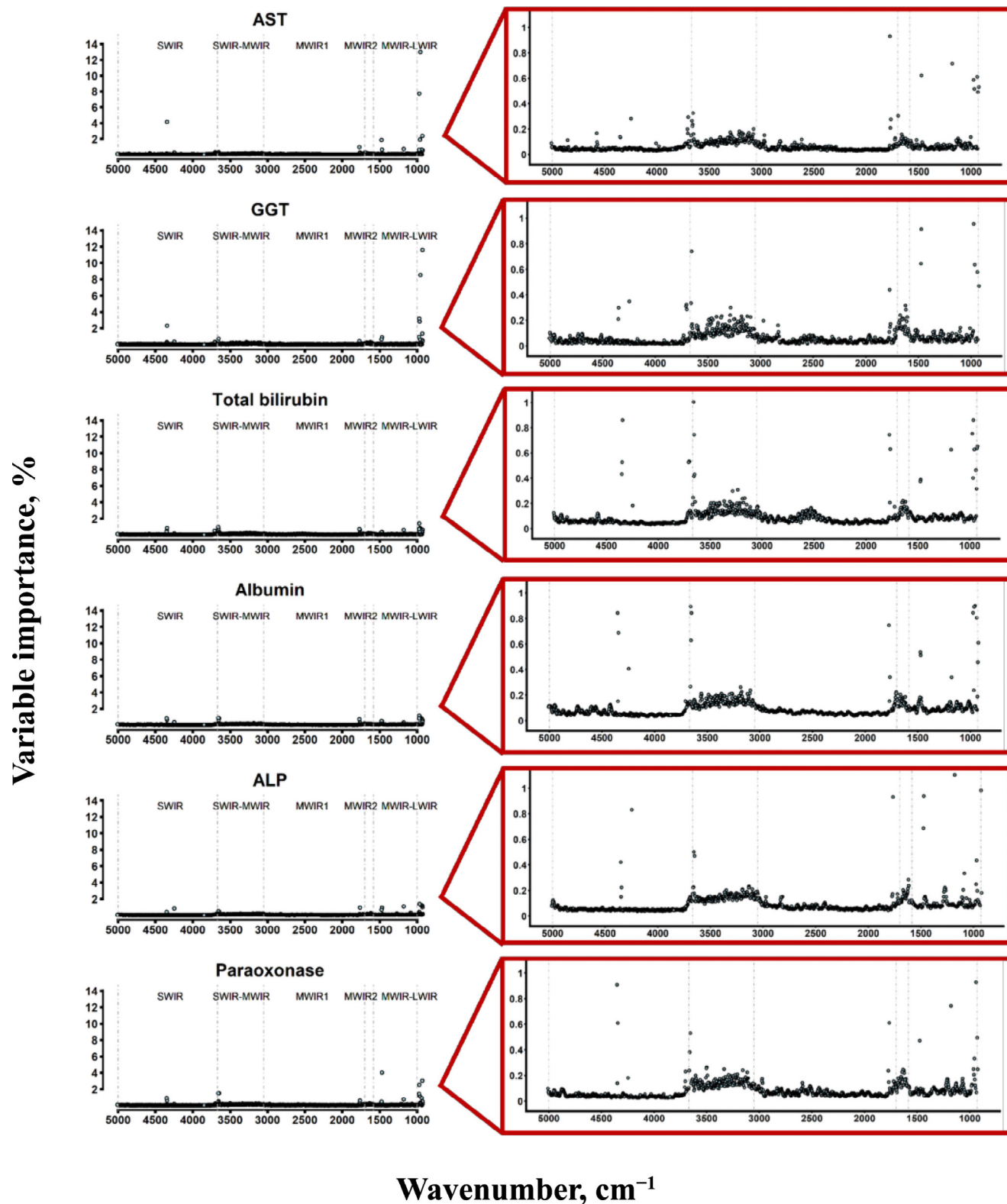


Figure 3. Variable importance values for the best prediction model selected by the automatic machine learning tool across the entire Fourier-transform mid-infrared spectrum (1,060 wavelengths) for blood metabolites related to liver function and hepatic damage. In the red square is a magnification of the lowest part of the y -axis ($<1.5\%$) to appreciate each wavelength's contribution. AST = aspartate aminotransferase; SWIR = short-wavelength infrared; MWIR = mid-wavelength infrared; LWIR = long-wavelength infrared; GGT = γ -glutamyl transferase; ALP = alkaline phosphatase.

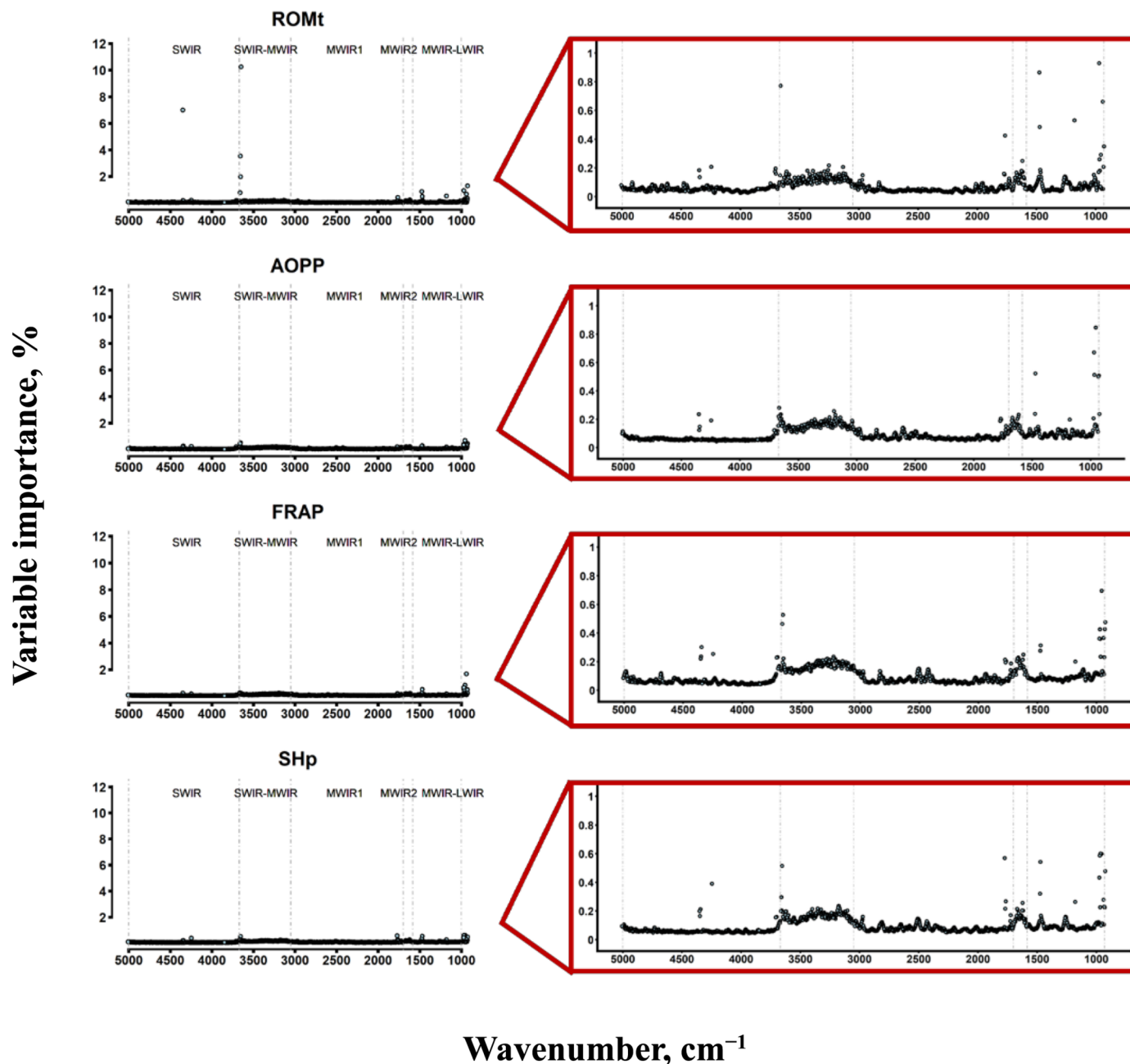


Figure 4. Variable importance values for the best prediction model selected by the automatic machine learning tool across the entire Fourier-transform mid-infrared spectrum (1,060 wavelengths) for blood metabolites related to oxidative stress. In the red square is a magnification of the lowest part of the y -axis ($<1.5\%$) to appreciate each wavelength's contribution. ROMt = total reactive oxygen metabolites; SWIR = short-wavelength infrared; MWIR = mid-wavelength infrared; LWIR = long-wavelength infrared; AOPP = advanced oxidation protein products; FRAP = ferric reducing antioxidant power; SHp = total thiol groups.

3,708 and 3,650 cm^{-1} made a relevant contribution to the model's predictive ability for albumin, BILt, and paraoxonase, explaining between 0.5% and 1.5% of the importance of these variables.

The most informative region for oxidative stress metabolites was once again between 975 and 925 cm^{-1} (Figure 4). Last, wavelengths of 3,654 and 3,650 cm^{-1}

represented between 0.5% and 10.2% of the importance of AOPP, ROMt, and SHp.

Regarding the category of inflammatory status indicators, wavelengths between 3,658 and 3,650 cm^{-1} contributed from 0.7% to 8.6% of the model's predictive ability. For total proteins, globulins, myeloperoxidase, and ceruloplasmin, we found a clear contribution

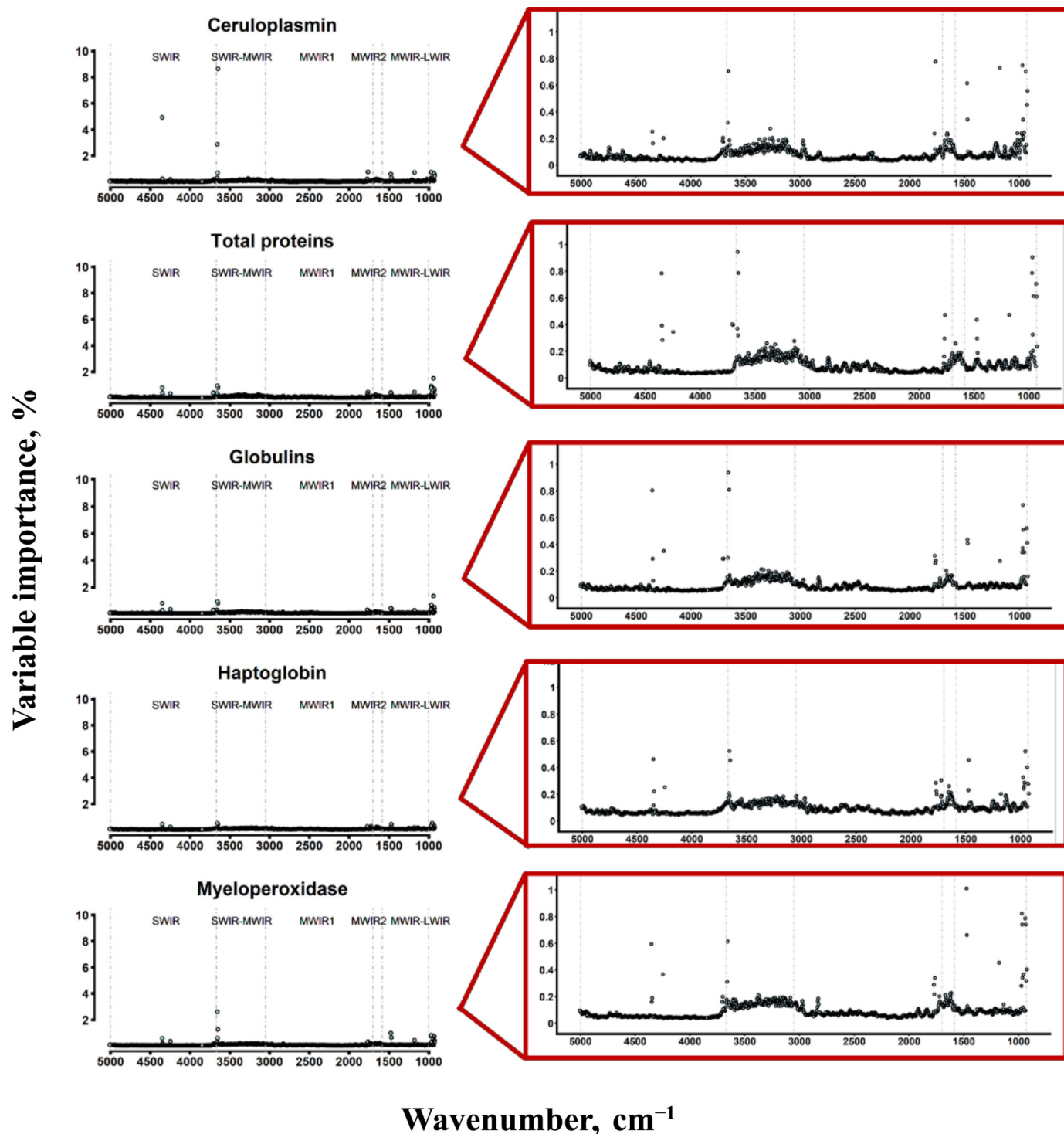


Figure 5. Variable importance values for the best prediction model selected by the automatic machine learning tool across the entire Fourier-transform mid-infrared spectrum (1,060 wavelengths) for blood metabolites related to immune response and innate immunity. In the red square is a magnification of the lowest part of the y -axis ($<1.5\%$) to appreciate each wavelength's contribution. SWIR = short-wavelength infrared; MWIR = mid-wavelength infrared; LWIR = long-wavelength infrared.

of wavelength $4,351\text{ cm}^{-1}$, explaining from 0.6% (in myeloperoxidase) to 4.9% (in ceruloplasmin) of their importance. Ceruloplasmin was also associated ($>0.6\%$

contribution) with wavelengths between $1,770$ and $1,470\text{ cm}^{-1}$ in the MWIR-LWIR transition region (Figure 5). Because the hematochemical parameter haptoglobin

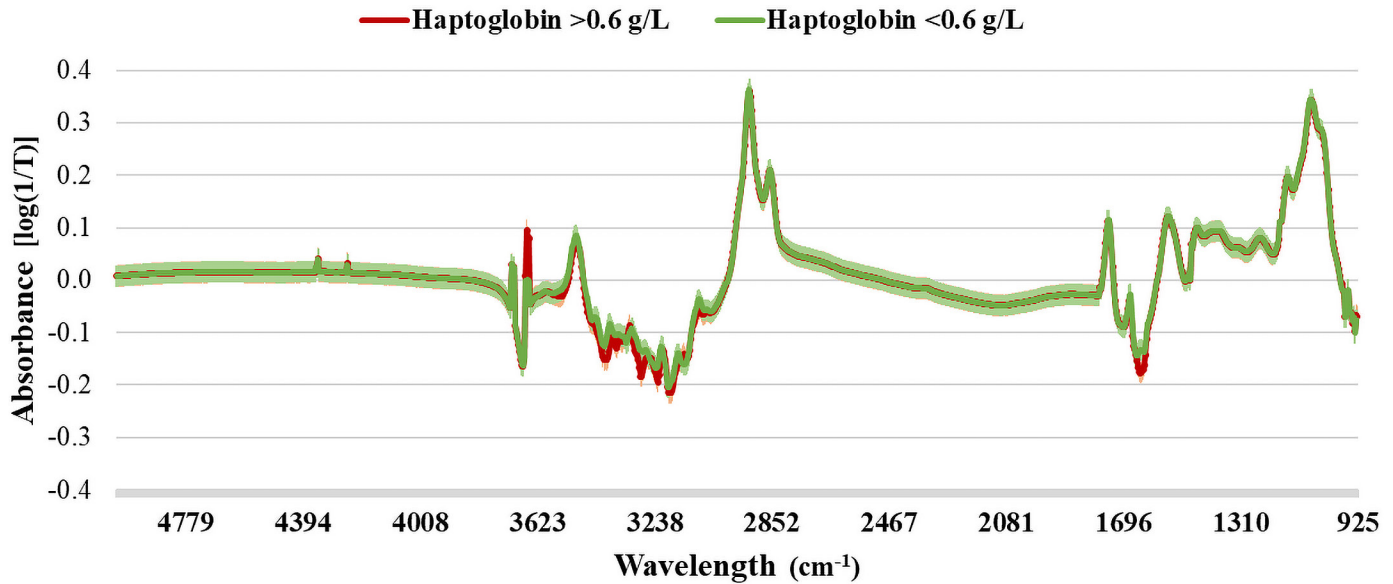


Figure 6. Comparison of the entire Fourier-transform mid-infrared spectrum (1,060 wavelengths) between cows with high serum levels of haptoglobin (red line) and the rest of the population (green line). Light-green and orange halos show the SD.

had a high frequency of individuals with altered values, combined with good TPP precision in Q75, we compared the milk spectrum of individuals with high serum levels (>0.6 g/L, $n = 172$) with the spectrum of the rest of the population ($n = 1,016$) and found that wavelengths 4,351, 3,658, and 3,650 cm^{-1} differed most significantly (Student t -test; $t > |15|$, $P < 0.001$; Figure 6).

Last, the wavelengths that made the greatest contribution to blood mineral prediction (range of explained importance, 0.5% to 16.6%) were located in the SWIR-MWIR transition region from 3,708 to 3,650 cm^{-1} , and in the MWIR2 region from 1,773 to 1,472 cm^{-1} , with a wavelength of 1,179 cm^{-1} making a particular contribution to Ca, Cl, and Zn (Figure 7).

DISCUSSION

The data used in this study were collected from herds under different management conditions and located in various regions of northern Italy, which allowed us to design independent CV scenarios. With regard to the data set used for BHB prediction, an additional source of variability was provided by a group of multibreed traditional farms located in a mountain area (Sturaro et al., 2013), which were characterized by lower average milk production compared with farms located in the Veneto and Emilia Romagna regions (Table 1). Indeed, the use of different breeds (and therefore greater variability) has been shown to increase the predictive ability of milk FTIR spectra

when applied on serum BHB (Mota et al., 2021a). The latter farms were also under different management systems, especially with regard to the implementation of health measures (e.g., differences in the prevalence of metabolic disorders and clinical/subclinical mastitis). Although we excluded cows with clinical diseases from the trial, we included cows with various degrees of putative subclinical conditions, as shown by the proportion of individuals exceeding the physiological thresholds for blood traits (e.g., haptoglobin and urea; Supplemental Table S1).

Because our study included cows at all stages of lactation (median DIM of the population, 184 d), average BHB blood levels were lower and more variable than in the previous studies (Pralle et al., 2018; Grelet et al., 2019; Luke et al., 2019). This could also explain the low prevalence of hyperketonemia in the investigated data set, because only 10% of the cows were in the first 50 d postpartum. Indeed, a limitation of our study might be the low number of individuals in the early lactation phase, resulting, consequently, in a low prevalence of hyperketonemia and high NEFA levels. However, recent studies have highlighted that BHB alteration persists and is observable until the peak of lactation (Couperus et al., 2021). In addition, late lactation and dry-off phases heightened attention as predisposing moment for the early start of metabolic diseases (Mezzetti et al., 2020; Premi et al., 2021), with lower and more subtle thresholds of BHB that need to be monitored (0.5 to 1 mmol/L; Lisuzzo et al., 2022).

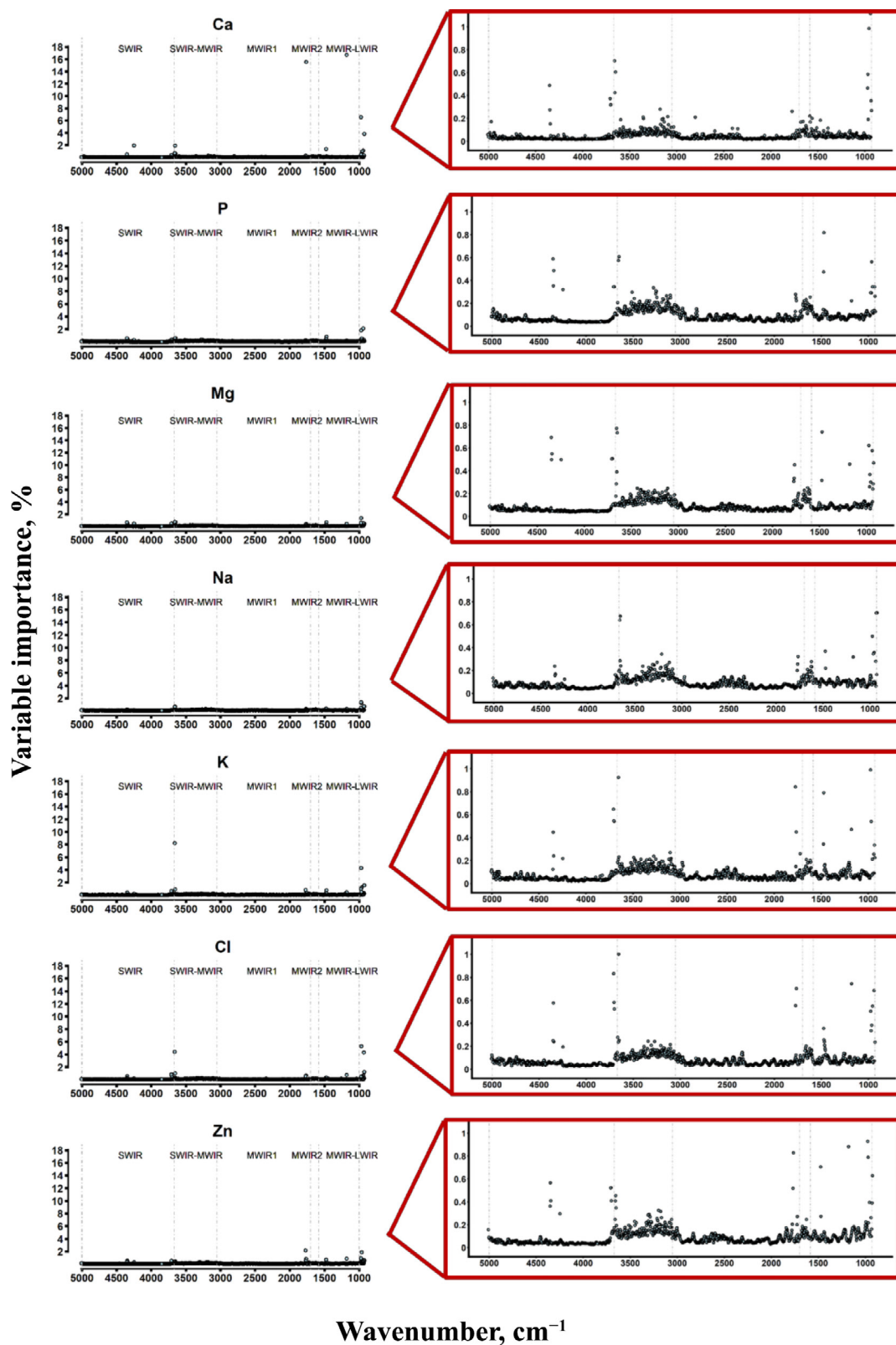


Figure 7. Variable importance values for the best model selected by the automatic machine learning tool across the entire Fourier-transform mid-infrared spectrum (1,060 wavelengths) for blood minerals. In the red square is a magnification of the lowest part of the y -axis (<1.5%) to appreciate each wavelength's contribution. SWIR = short-wavelength infrared; MWIR = mid-wavelength infrared; LWIR = long-wavelength infrared; T = transmittance.

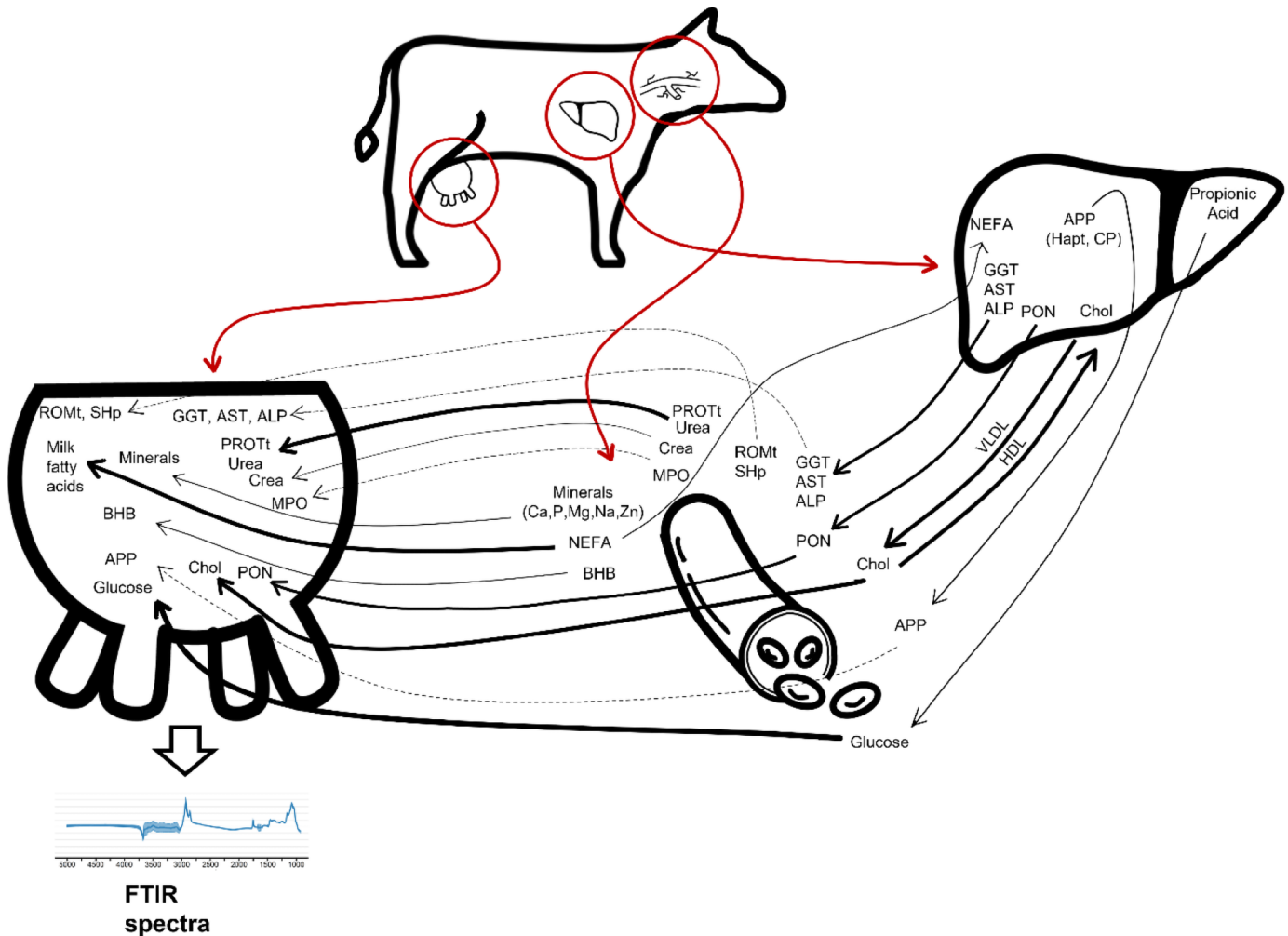


Figure 8. Origin/bypass of substances present in the mammary gland of dairy cows captured with Fourier-transform mid-infrared (FTIR) spectroscopy and relationships with bloodstream and liver (modified from Giannuzzi et al., 2022). The thick arrows represent gross transfer between compartments during lactation. In the mammary gland, the metabolites involved in these processes are mainly derived from blood flow. The thin arrows denote minor fluxes. Dashed lines represent fluxes of metabolites originated in part from the bloodstream and secreted in part directly by the mammary gland. ALP = alkaline phosphatase; APP = acute-phase proteins; AST = aspartate aminotransferase; Chol = cholesterol; CP = ceruloplasmin; Crea = creatinine; GGT = γ -glutamyl transferase; HDL = high-density lipoproteins; Hapt = haptoglobin; MPO = myeloperoxidase; NEFA = nonesterified fatty acids; PON = paraoxonase; PROTt = total proteins; ROMt = total reactive oxygen metabolites; SHp = total thiol groups; VLDL = very low-density lipoproteins.

Model Performance

To develop our prediction equations, we chose the autoML function of the h2o R package (R Foundation for Statistical Computing), which used penalized regression (EN), ML techniques (DRF, GBM, XGB, and ANN), and a stacking ensemble approach to learn the best combination of different ML techniques. In addition, the use of distribution-free approaches could improve predictive ability for those traits with low-variation ranges (e.g., glucose, Na, Cl, Ca) or a skewed distribution (e.g., NEFA, BHB). Among the base learning approaches, the greater prediction accuracies obtained by EN could be attributed to the combination

of λ_1 (RR) and λ_2 (LASSO) penalties, which control the degree of shrinkage applied to the FTIR wavelengths, enforcing many coefficients to exactly zero or close to zero (Zou and Hastie, 2005; Hastie et al., 2009). Several authors have evaluated ML methods, demonstrating their superiority in prediction accuracy against PLS regression (Pralle et al., 2018; Soyeurt et al., 2020; Mota et al., 2021b). Blood metabolites have extremely different chemical structures and data distributions, and they are affected by different factors (e.g., hormones, environment, feeding). Therefore, an individual ML technique with a specific learning structure may show a limited ability to uncover the complex relationship between blood metabolites and milk FTIR predictors.

The stacking ensemble algorithm combined with milk near-infrared spectra have already shown a significant improvement in accuracy for cheese-making-related trait predictions (Mota et al., 2022) and for blood traits (Giannuzzi et al., 2022). Similarly, the greater prediction accuracy overall achieved by the stacking ensemble in our study may be related to its flexibility in exploiting the combination of different ML model structures (Wolpert, 1992).

Hematocrit had poor predictability, with all the algorithms evaluated in agreement with results observed by Giannuzzi et al. (2022) using milk near-infrared spectra. In addition to the very low variability of this trait in the populations, hematocrit is not an hematochemical parameter, but rather a cellular measurement associated exclusively with blood, with no apparent relation to milk components. No comparable studies have yet been carried out using milk FTIR spectra to predict hematocrit, so further investigation is needed to confirm this finding. Given its negligible predictability, hematocrit is not discussed further.

Evaluating diverse CV scenarios with different dependencies among the training and validation sets is a crucial aspect to define a model's performance. Specifically, applying a more conservative CV with low dependence between training and validation sets, such as CV_h in our study, is fundamental to a realistic assessment of prediction accuracy. Consistent with previous findings (Wang and Bovenhuis, 2019), the CV_h scenario had a lower predictive ability than the CV_r scenario, but the reduction of accuracy was slight, confirming the robustness of the analysis ($R^2 > 0.50$), with the exceptions of urea, FRAP, and haptoglobin. As mentioned earlier, with the aim of achieving maximal representativeness of a northern Italy dairy herd panorama, we included in the investigated population different herds in terms of management and feeding. As a result, the hematochemical traits that are more prone to changes in relation to feeding or health conditions were difficult to predict among herds. This is the case with haptoglobin, for which the prevalence of its alteration was herd dependent, and urea, for which there was a high variability of diets among herds. The same considerations for urea could be made with FRAP, because uric acid is estimated to contribute ~60% to the FRAP value in plasma (Benzie and Strain, 1996).

Predictive Ability and the Biological Link Between Milk and Blood

Various blood-derived compounds are present in very low concentrations in normal milk. The exchange of soluble and cellular components between blood and milk in the mammary gland is controlled by the blood–milk

barrier (Wellnitz and Bruckmaier, 2021). This semipermeable barrier regulates the concentrations of desired milk components, such as lactose, milk proteins, and K, and prevents the uncontrolled transfer of specific blood constituents into the milk (Linzell and Peaker, 1971). Figure 8 provides an overview of the exchanges between blood and milk with regard to the blood components examined in our study.

Inflammation and Innate Immunity-Related Metabolites. The biological category consisting of the inflammation and innate immunity-related metabolites exhibited the best predictive ability. The presence of blood-derived proteins in milk is well documented, although the relationships involved in active and passive transfer are complex (Wall et al., 2015). In cows, ceruloplasmin and haptoglobin are major acute-phase proteins that are known to be diffused from blood leakage into the milk, but are also produced endogenously in the mammary gland by milk leukocytes and epithelial cells during local inflammatory processes (Nielsen et al., 2004; Upadhyaya et al., 2016). Myeloperoxidase is a lysosomal peroxidase enzyme released mainly by activated neutrophils during first-line inflammatory responses, and its presence in milk has been associated with ongoing infections (Cooray, 1994). The fact that these molecules are present in milk and blood in the same form can easily explain their good predictability. In fact, the chemical structure of the various amino acids is captured at specific wavelengths, but the 3-dimensional structure of a protein, or the presence of a cofactor, could also affect the absorption pattern of the spectrum. The MWIR2 region (particularly between 1,650 and 1,700 cm^{-1}) is highly informative of protein secondary structure (Barth, 2007). Within this category, TPP for classification of extreme values was good for both total proteins and globulins. Previous findings from a smaller set of data with a similar range of variation showed low predictive ability for albumin and globulins using PLS on milk FTIR spectra (Luke et al., 2019). Using ML algorithms on a larger data set would probably improve predictive ability, corroborating the robustness of ML methods, particularly ensemble learning, for the prediction of imbalanced phenotypes (Fan et al., 2011). Of note, haptoglobin showed a good TPP value for Q75. In milk, the source of haptoglobin during inflammatory processes has been shown to be one or a combination of endogenous production in mammary gland tissue, migrating neutrophils, or bloodstream leakage resulting from the permeability of the blood–milk barrier (Hiss et al., 2004; Thielen et al., 2007). In our data set, haptoglobin had a bimodal distribution, with a consistent number of records greater than the physiological threshold of 0.30 g/L, suggesting a low degree of systemic inflammation in the cows in-

vestigated. It could be that the presence of animals with greater values of haptoglobin allowed the model to perform well in classifying individuals in Q75, even with a moderate prediction ability. This result shows the importance of evaluating prediction accuracy in association with the class of interest (i.e., below/above known risk thresholds), but also suggests the potential reliability of predicted haptoglobin as an indicator of systemic inflammatory disorders as well as local affections. Indeed, comparison of the individuals with high serum haptoglobin concentrations with the rest of the population showed that specific wavelengths within the milk spectrum were able to differentiate between cows with low and high levels of serum haptoglobin (Figure 6). These wavelengths are $\sim 3,600\text{ cm}^{-1}$ and are related to amines and amide A, and derive mainly from N–H or hydroxyl (O–H) stretching/bending vibrations (Ji et al., 2020). Differences in absorbance in protein amide bands indicate conformational changes to protein, which is a known phenomenon for haptoglobin in inflammatory conditions in humans (e.g., haptoglobin glycosylation; Motooka et al., 2021).

Oxidative Stress Metabolites. Oxidative stress metabolites constituted the category with the second-best predictive ability, with the greatest R^2 value achieved by ROMt and SHp. To date, no studies have investigated the predictability of these parameters using milk FTIR spectra. Nonetheless, FTIR spectra have proved useful in monitoring oxidative stress modification in blood, as this method is able to detect the presence of their functional groups (Bujok et al., 2019). Oxidant/antioxidant imbalance (and, consequently, oxidative stress) has been linked to metabolic disorders in dairy cows (Abuelo et al., 2019; Gabai et al., 2019; Zheng et al., 2021). Plasma thiols, reactive oxygen metabolites, and AOPP are present and detectable in milk (Abeni et al., 2008; Gutierrez et al., 2018; Gabai et al., 2019), and correlate with the oxidative stress status of the individual, according to its capacity for homeostatic balance (Rizzo et al., 2013). Our findings show that it is possible to obtain reliable predictions of blood oxidant–antioxidant status from milk using FTIR spectra, given the high TPP for ROMt and SHp in Q75, which represents the class of major interest in evaluating oxidative status imbalance. The ability to identify individuals with high oxidant or antioxidant activity, or their imbalance, could be crucial in the early detection of energetic and metabolic impairments in high-yielding dairy cows.

Energy-Related Blood Metabolites. The category of energy-related blood metabolites had the third-best predictive ability. Within this category, glucose and urea achieved good predictive ability and precision in both Q25 and Q75. Blood urea levels reflect the dietary

intake of CP, from digestive use to milk protein secretion and N urinary excretion (Kessler et al., 2020). The diets of animals on high-producing dairy farms commonly contain CP at levels exceeding 16% to ensure maximum milk output (Colmenero and Broderick, 2006), as was the case for the herds we investigated (Giannuzzi et al., 2021). Milk urea is highly correlated linearly with blood urea (Butler et al., 1996), and several studies have shown the close interplay between blood and milk in urea transfer (Spek et al., 2012, 2016). It follows that a high coefficient of determination is expected. Consistent with previous studies (Benedet et al., 2019; Luke et al., 2019; Ho et al., 2021), we observed a moderate degree of reduction in prediction ability when testing more conservative scenarios (R^2 of $CV_h = -11\%$ compared with CV_r). This is probably a result of the intrinsic nature of circulating urea, which reflects ruminal ammonia clearance, and the efficiency of protein and N metabolic use being the result, in part, of the feeding management of individual herds and related, in part, to relative individual protein efficiency (Liu and VandeHaar, 2020).

Aside from being the precursor for lactose synthesis, glucose is present in milk (Smith et al., 1983). Because of the relevant association between hypoglycemia and its detrimental effect on milk production (Ruoff et al., 2017), precise prediction of Q25 is a promising step toward the future possibility of using blood glucose predictions in milk for early detection of subclinical ketosis.

With regard to the other indicators of metabolic impairment, blood BHB is considered the most reliable marker of hyperketonemia, and we obtained a moderate prediction accuracy ($0.45 < R^2 < 0.61$), which is similar to several previous results (Pralle et al., 2018; Luke et al., 2019; Ho et al., 2021), but less than others (Benedet et al., 2019; Grelet et al., 2019). As discussed earlier, there was a low prevalence of elevated serum BHB concentrations in our data set, and this probably reduces the predictive ability for higher BHB concentrations, as reported by Luke et al. (2019).

Serum NEFA were predicted accurately with a greater coefficient of determination than previously developed prediction equations (Grelet et al., 2019; Luke et al., 2019; Ho et al., 2021). Even if the variability of NEFA in our study was in the physiological range, Q75 was predicted accurately, with a good TPP value. This trait was highly variable in our data set (75% CV), particularly in Q75, and this was probably responsible for the high precision of the prediction equation we developed. Other studies that include high blood NEFA concentrations in the data set (in the pathology range) are needed to validate further the precision of these prediction equations for the early diagnosis of

negative energy balance. In our study, only a few wavelengths contributed strongly ($>0.5\%$) to the variable importance of blood NEFA prediction, and as Figure 2 shows, the regions that contributed the most to prediction accuracy were those $\sim 3,000$, $1,700$, $1,000$, and 960 cm^{-1} , corresponding to relevant fat absorption bands: fatty acid unsaturation, C=O stretch, C–O stretch, and trans-C–H stretch vibrations, respectively (Afseth et al., 2010; Aernouts et al., 2020).

This is the first work to evaluate the predictive ability of milk FTIR spectra for blood creatinine. Creatinine is a more sensitive indicator of the renal glomerular filtration rate in ruminants than urea, and gives the magnitude of N excretion when measured in urine and, to a minor extent, in milk (Smith, 2015). In fact, creatinine is released into the milk from the bloodstream, and can be detected and measured there (Müller et al., 2021). We found a good predictive ability for this trait, highlighting the potential of using FTIR spectra for noninvasive detection of creatinine. Further improvement in the prediction equations is needed to confirm this finding.

Metabolites Related to Hepatic Function and Hepatic Damage. Within the group of metabolites related to hepatic function and hepatic damage, predictive ability was moderate overall, except for albumin, which was predicted accurately in both CV scenarios. Physiologically, albumin from blood serum represents up to 1% of the total N in milk (Fox and McSweeney, 1998). In inflammatory conditions, enhanced capillary permeability leads to proteins passing from blood to the udder, increasing the concentration of serum albumin in the milk (Poutrel et al., 1983). As for inflammatory proteins, the regions $3,600$ to $3,050$ and $1,650$ to $1,550\text{ cm}^{-1}$ make an important contribution to the importance of the albumin variable, and correspond to relevant protein absorption bands—namely, primary and secondary amines (N–H stretch/bend vibration), amide I (C=O stretch and C–N stretch vibrations), and amide II (N–H bend and C=N stretch vibrations; Krimm and Bandekar, 1986). Also, TPP values for albumin in Q25 and Q75 were $\sim 70\%$, revealing good prediction accuracy in both cases, and highlighting the potential to use milk FTIR spectra for accurate prediction of albumin.

On the contrary, ALP, AST, and GGT showed a moderate predictive ability in both CV scenarios. Hepatic enzymes are present physiologically in cows' milk as a consequence of spontaneous diffusion of low-molecular-weight enzymes from plasma, active secretion by the mammary gland epithelium, or release after disruption of the mammary leukocyte population. Alkaline phosphatase and GGT are reported to have much greater activity in milk than in blood serum, and fluctuate not only with increased plasma activity, but also in relation

to stage of lactation, seasonality, milk yield, and mammary gland health (Calamari et al., 2005). However, a relationship has been found between impaired energetic metabolism and blood and milk AST, ALP, and GGT, with mild hepatic lesions associated with an increase in the enzymes in both matrices (Liu et al., 2012; Calamari et al., 2015). Given all this, the potential to predict serum hepatic enzymes accurately from milk is interesting and deserves further investigation.

Total bilirubin was predicted accurately with CV_r, with slightly lower results for CV_h, but with a high prediction accuracy in Q75. Total bilirubin is a signal of clearance of the breakdown products of heme catabolism, and an increase in bilirubinemia is a sign of abnormal or reduced liver function (Graugnard et al., 2012; Zhou et al., 2016). Total bilirubin has been proposed frequently as a specific and sensitive marker for the risk of hepatic dysfunction—and, consequently, ketosis—and has been a key component in calculating the liver functionality index (Bertoni and Trevisi, 2013; Sun et al., 2015). In light of this, the use of milk FTIR spectra to predict plasma BILt, together with other parameters such as albumin and cholesterol, might be a relatively simple, sensitive, specific, and inexpensive method for assessing liver function.

Minerals. The category of plasma minerals showed the poorest predictability, an expected finding for 2 main reasons. First, the minerals in this data set had low variability compared with other blood metabolites (an average CV of 9%). Second, minerals do not have specific absorption bands in the infrared spectrum, so their prediction might therefore be related to their occurrence in other milk organic compounds, such as proteins and organic molecules (Huang et al., 2009). More precisely, Ca predictions are known to be associated with the protein content of milk, and the predictability of organic P is related to casein micelles as calcium phosphate nanoclusters; in contrast, inorganic P and Mg are freely distributed in the soluble milk serum phase (Gaucheron, 2005). The predictive ability of Ca, P, and Mg using the prediction equations we developed was moderate, but is nonetheless greater than that obtained in a previous study (only Ca and Mg; Luke et al., 2019).

Prediction of Na, K, and Cl could, however, be associated with the prediction of milk components related to osmotic equilibrium maintenance, such as lactose (Zaalberg et al., 2021). In the case of Zn, it is bound mainly to milk components, such as caseins, whey proteins, citrate, and inorganic phosphate, and as a cofactor of several metalloenzymes (McCall et al., 2000). Blood Na and Cl showed high prediction accuracy with CV_r, at levels of precision ranging from moderate to good in the 2 extreme classes. Sodium, Cl, and Zn are

connected with the immune system and inflammatory status. The mammary gland is highly sensitive to osmotic changes, and permeability to Na and Cl increases during inflammation (Pezeshki et al., 2011). On the other hand, there may be a reduction in Zn during intense Zn-mediated antioxidant and reducing activities (Olechnowicz et al., 2018). The potential of using prediction equations for rapid, simple quantification of the variations in these minerals merits further investigation with a larger population, which would increase the variability in these traits.

CONCLUSIONS

The prediction equations for serum metabolites using milk FTIR spectra varied according to blood parameter, but were, on the whole, accurate and show promise for large-scale monitoring. Specifically, EN and stacking ensemble algorithms exhibited the best predictive ability for most of the blood parameters, outperforming the other ML methods and PLS regression. As expected, we observed a putative relationship between high amounts of them in milk and their good predictability (e.g., proteins and enzymes). However, FTIR spectra seem able to capture indirect information associated with blood compounds that are only detectable at trace concentrations in milk (e.g., minerals, BILt), yielding a satisfactory predictive ability. Therefore, the complex matrix of milk is probably informative of variations in the metabolic status of individual cows, regardless of the presence of specific marker compounds.

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