

# Clinical biochemical and hormonal profiling in plasma: a promising strategy to predict growth hormone abuse in cattle

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**Abstract** Recombinant bovine somatotrophin (rbST) is widely used in some countries to increase milk production. Since 1994, both marketing and use of this substance have been prohibited within the European Union. In this context, the targeted plasma biochemical and hormonal profiling was assessed as a potential screening strategy to highlight rbST (ab)use in cattle. Twenty-one routinely measured clinical blood parameters, representative of main biological profiles (energetic, proteic, etc.), were measured in the plasma of six lactating cows before and after rbST treatment throughout a 23-day study period. Appropriate multivariate statistical analyses [principal component analysis (PCA) and orthogonal partial least square (OPLS)] enabled discriminating animal samples before and after treatment (days 0 vs. 2 to 9,  $P=2.10^{-9}$ ) and highlighted the five most relevant blood parameters in this discrimination. Based on each five-analyte contribution, a simple mathematically weighted equation was suggested to predict the status of samples. A suspicious threshold was proposed, and the model was further tested with the status prediction of the supplementary samples from untreated ( $n=20$ ) and treated cows ( $n=22$ ). The calculated false-positive (10 %) and false-negative (4.5 %) rates were in accordance with the

EU requirements for screening methods. Although the model needs to be further validated with additional samples, such targeted plasma biochemical and hormonal profiling already appears as a potential promising screening strategy to highlight rbST (ab)use in cattle.

**Keywords** Bovine recombinant somatotrophin · Blood parameters · Screening method · Biomarkers

## Introduction

Bovine somatotrophin (bST), also called bovine growth hormone (bGH), is an important endocrine factor for normal growth and maintenance of all tissues, reproductive functions and lactation [1]. Since the 1980s and the breakthrough of recombinant DNA technologies, recombinant bovine somatotrophin (rbST) has become commercially available in large quantities and therefore has been used to increase milk production for dairy cattle [2]. The European Union decided to prohibit its use and its marketing in 1994 (European Council decision 99/879/EC [3]). Nevertheless, the risk of illegal distribution and use within the EU cannot be excluded and efficient analytical strategies are required to monitor such misuse [4].

So far, different analytical strategies have been developed to detect rbST abuses in bovines [4]. For confirmatory purposes, the unambiguous identification of rbST in matrices such as blood has become recently possible thanks to efficient purification procedure combined with the use of recent mass spectrometry instruments [5]. Nevertheless, the detection of rbST at a trace level in these matrices requires quite a few steps of sample preparation, the use of expensive instruments, and even so, rbST can only be detected within 4 days after the last injection [5]. Thus, and according to the European legislation

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in force in food safety arena (Decision 2002/657/EC [6]), affordable and efficient screening methods exhibiting the capability for a high sample throughput are required in order to sift a large number of samples for potential non-compliant results. One recognized analytical approach, complying with these requirements, is based on the detection of anti-rbST antibodies in the blood by immunoassays, thus allowing the identification of rbST-treated cows for a long time period [7]. However, the immune response requires some days to occur, and therefore, this screening method is efficient only after more than 1 week post-treatment. Other blood-related biological markers of rbST administration in bovines (i.e. insulin-like growth factor I (IGF-I), IGF-binding protein 2 (IGFBP2) and osteocalcin) were reported and used in combination with anti-rbST antibodies to screen rbST-treated animals [8]. Although particularly promising, the authors reported a detection window starting only after the second rbST injection.

Another promising possibility to detect rbST abuse may be based on the study of the physiological disorders induced by such a treatment through the profiling of routinely measured clinical blood parameters (e.g. urea, insulin, cholesterol, etc.). Blood concentrations of these compounds are related to breed and physiological conditions (e.g. age, reproductive status, disease, etc.) and therefore reflect the general status of the animals [9, 10]. Treatment with anabolic compounds has an impact on blood parameters, and atypical concentrations of

these analytes have already been shown after anabolic steroid administration in bovines [11, 12]. Moreover, a recent study reported that targeted clinical metabolic profiling of cattle sera could be used as a test for predicting steroid misuse [13]. RbST has direct or indirect effects (mediated by IGF-I) on several organs such as the muscle, liver, bone, etc., and therefore on many biological mechanisms such as lipogenesis, carbohydrate metabolism, protein metabolism, etc. [1, 14, 15]. For example, after rbST injection, a measurable rise in non-esterified fatty acid (NEFA) has been reported as a consequence of the increased use of fat as an energy source [14]. Thus, the administration of rbST could be expected to induce specific blood clinical parameter profile which could be used as a predictive tool to investigate rbST treatment in dairy cattle.

In the present study, 21 routinely measured clinical blood parameters were analyzed in six lactating cows before and after rbST treatment throughout a 23-day study period. The 21 plasma analytes are representative of main biological profiles (e.g. hepatic, energetic, protein, etc.) and are presented in Table 1. The overall aim of this preliminary study was to assess the potential of targeted clinical blood parameters, selected and combined by appropriate statistical tool, as a relevant approach to detect rbST (ab)use; careful precautions were taken regarding the interpretation of the results (number of animals, age, etc.).

**Table 1** Analytical methods and performances for the 21 targeted blood parameters

Profiles	Targeted blood parameters	Analytical method	Linearity	Intra-assay variation (RSD %)	Inter-assay variation (RSD %)
Hepatic	Alanine transaminase (ALT)	IFCC without P5'P	5–700 U L <sup>-1</sup>	0.6	2.6
	Aspartate transaminase (AST)	IFCC without P5'P	5–700 U L <sup>-1</sup>	1.4	1.9
	Alkaline phosphatase (ALP)	IFCC	5–1200 U L <sup>-1</sup>	0.9	2.1
	Gamma-glutamyl transferase (GGT)	Standardized to Szasz	3–1200 U L <sup>-1</sup>	1.5	1.9
	Creatine kinase (CK)	IFCC	7–2000 U L <sup>-1</sup>	2.3	2.5
	Lactate dehydrogenase (LDH)	IFCC	10–1000 U L <sup>-1</sup>	1.3	2.7
	Insulin (INS)	Immunochemiluminescence	14–2165 pmol L <sup>-1</sup>	6.4	8.0
	Bilirubin [direct (DB), indirect (IB), total (TB)]	DPD	DB, 1.5–291.0 μmol L <sup>-1</sup> ; TB, 1.7–650.0 μmol L <sup>-1</sup>	DB, 2.9; TB, 2.7	DB, 4.0; TB, 4.5
Energetic	Glucose (GLU)	Hexokinase	0.1–41.6 mmol L <sup>-1</sup>	0.7	1.2
	Non-esterified fatty acid (NEFA)	Colorimetric	0.07–2.24 mEq L <sup>-1</sup>	4.7	4.5
	Cholesterol (COL)	Enzymatic CHOD-PAP	0.1–20.7 mmol L <sup>-1</sup>	2.1	2.6
	Triglycerides (TG)	Enzymatic GPO-PAP	0.1–10.0 mmol L <sup>-1</sup>	1.1	1.9
	β-Hydroxybutyrate (β-OHB)	Kinetic enzymatic	0.10–5.75 mmol L <sup>-1</sup>	3.8	5.2
Proteic	Albumin (ALB)	BCG	2–60 g L <sup>-1</sup>	0.7	1.0
	Urea (UR)	UV kinetic urease	0.5–40.0 mmol L <sup>-1</sup>	2.1	3.4
	Total protein (TP)	Biuret	2–120 g L <sup>-1</sup>	1.1	1.5
	Creatinine (CREA)	Enzymatic PAP	5–2700 μmol L <sup>-1</sup>	1.1	1.2
	Globulin (GLO)	Calculated parameter	–	–	–
Growth	Insulin-like growth factor I (IGF-I)	Immunochemiluminescence	5.5–1380.0 ng mL <sup>-1</sup>	5.8	6.3

## Materials and methods

### Plasma parameter analysis

Blood parameters (Table 1) were determined using commercial kits applied to an automated clinical chemistry analyser (Cobas C501; Roche Diagnostics, Mannheim, Germany). Commercial kits for all parameters were provided by Roche Diagnostics, with the exception of NEFA and  $\beta$ -hydroxybutyrate ( $\beta$ -OHB) determined respectively with a colorimetric method and a kinetic enzymatic method with kits produced by Randox Laboratories Ltd., Crumlin, UK. Insulin and IGF-I determination was performed with a dedicated commercial kit (Siemens Healthcare Diagnostics, Gwynedd, UK) applied to an automated chemiluminescent system (Immulin One; Siemens Healthcare Diagnostics).

Performances of the method were determined and are reported in Table 1. The intra-assay variation, expressed as the relative standard deviation (RSD), was calculated by measuring two pools of samples ten times in a single analytical run, with low and high concentrations of each analyte, respectively. The inter-assay variation was determined by analysis of the same pools in duplicate on five different days.

Samples were analyzed randomly in order to prevent analytical bias and to ensure that any highlighted differences between samples would only be due to biological factors and not to analytical variations.

### Animal experiments

A total of 48 plasma samples, collected from cows ( $n=25$ , repeated sampling at different times for some of them) presenting different ages (from 2 to 7 years) which had never been administered with rbST or before rbST administration, were used as control samples. Three animal experiments (A, B and C) involving a total of nine cows were performed in order to provide the study with blood samples from rbST-treated cows ( $n=105$ ). The sampling scheme of the control and treated samples is presented in Table 2. Besides the classical expected administration scheme (500 mg recombinant bGH (rbGH) every 14 days), the experimental design of the present study also included higher dosages (1 g rbGH once or twice) to intensify the biological responses of interest. All animals were fed classical diet for lactating cows, representative of common practices.

Experiment A involving six lactating cows of different ages (from 2 to 5 years) and stages of lactation (identified as A-1 to A-6) was performed. The animals received one subcutaneous injection of Lactatropin<sup>®</sup> (500 mg of rbST, slow-release formula) (Elanco, Eli Lilly, Bryanston, South Africa) on day 0. Blood samples ( $n=69$ ) were collected before injection on day 0, then at 5 h (H5) and 10 h (H10) after injection and then on day 1 (only for three animals) and days 2, 3, 4, 5, 7, 9, 16 and 23.

Experiment B involving two lactating cows at the age of 3 and 4 years (identified as B-1 to B-2, respectively) was implemented as follows: B-1 received one injection, while animal

**Table 2** Sampling scheme of the control and treated samples

Application	Denomination	Number of animals	Number of sampling	Breed	Sex	Age (years)	Time/day of sampling
<b>Control samples</b>							
Build the prediction model ( $Y$ threshold)	A-D0	6	6	Holstein	Female	2 to 5	D0
	Control 1	5	10	Holstein	Female	3	Twice at 3-day intervals
	Control 2	1	12	Holstein	Female	4	Several during 1 month
Test the proposed criterion	B-D0	2	4	Holstein	Female	3 and 4	D4 and D0
	C-D0	1	2	Holstein	Female	4	D1 and D0
	Control 3	1	5	Holstein	Female	3	Several during 1 month
	Control 4	9	9	Holstein	Female	2 to 7	Punctually
Total		25	48				
<b>Treated samples</b>							
Build the prediction model ( $Y$ threshold)	A	6	63	Holstein	Female	2 to 5	H5, H10, D1, D2, D3, D4, D5, D7, D9, D16 and D23
Test the proposed criterion	B	2	24	Holstein	Female	3 and 4	H4, D1, D2, D6, D9, D14 (twice: before and after the 2nd rbST treatment), D15, D17, D19, D30 and D32
	C	1	18	Holstein	Female	4	H4, H6, D1, D2, D3, D6, D8, D10, D14, D15, D20, D28, D29, D30 and D31
Total		9	105				

B-2 received two injections of Lactatropin® both on days 0 and 14. Blood samples ( $n=28$ ) were collected on days 4 and 0, then at 4 h (H4) after injection, then on days 1, 2, 6, 9 and 14 (before and just after the second injection) and then on days 15, 17, 19, 30 and 32.

Experiment C was conducted on one cow (identified as C-1) at the age of 4 years. Two doses of Lactatropin® were injected subcutaneously on day 0. Blood samples ( $n=20$ ) were collected on days 1 and 0, then at 4 h (H4) and 6 h (H6) after treatment, then on days 1, 2 and 3 (during the morning and the afternoon on D1 to D3) and then on days 6, 8, 10, 14, 15, 20, 28, 29, 30 and 31.

All the samples were collected in heparin tubes. Samples were then centrifuged, and supernatants were collected to obtain the plasma. Finally, the samples were stored at  $-20\text{ }^{\circ}\text{C}$  until analysis.

A descriptive model was built based on experiment A due to the following: (i) it typically reflected a pattern of rbST misuse (single dose of 500 mg of rbST, slow-release formula, during lactation) and (ii) it offered important biological variability (ages, stages of lactation). Samples from experiments B and C were used to test the descriptive model.

In this preliminary study, a relative reduced number of samples have been used since the method is not intended for a whole population in age/gender/feeding. Growth hormone misuse only relates to female lactating cows, exhibiting similar physiologies and conditions (age, health, silage-based feeding, etc.). Thus, variables such as gender, age, health status (only cows in good health are concerned by rbST treatment) and feed will have no or only minor impact on measured parameter variations. Moreover, the overall aim of this study was to evaluate the potential of targeted blood parameters to highlight rbST abuse, not to validate, at this stage, its applicability for a whole population.

The animal experiment A was performed at ENSAIA (Vandoeuvre-les-Nancy, France), the animal experiment B at CER Groupe (Marloie, Belgium) and the animal experiments C and D at Oniris (Nantes, France) in agreement with animal welfare rules currently in force in the different institutions and approved by their respective ethical committees.

## Data analysis

Multivariate statistical analyses [principal component analysis (PCA) and orthogonal partial least square (OPLS)] were performed using SIMCA P+ 13.0 (Umetrics AB®, Sweden). For all analyses, data were log transformed and scaled according to the Pareto method in order to prevent prevalence of some variables compared to the others. PCA was first applied as an unsupervised strategy in order to get visual representation of data variabilities. OPLS was secondly used to build a descriptive and predictive model allowing the discrimination between treated and untreated bovines. Permutation test was carried out

automatically using the software and provided reference distribution of the  $R^2/Q^2$  values, which hence indicates the statistical significance of these parameters (50 random permutations). S plot was finally used to reveal the contribution of each variable on the predictive component and therefore to highlight the most discriminating blood parameters.

## Results and discussion

### Method performances

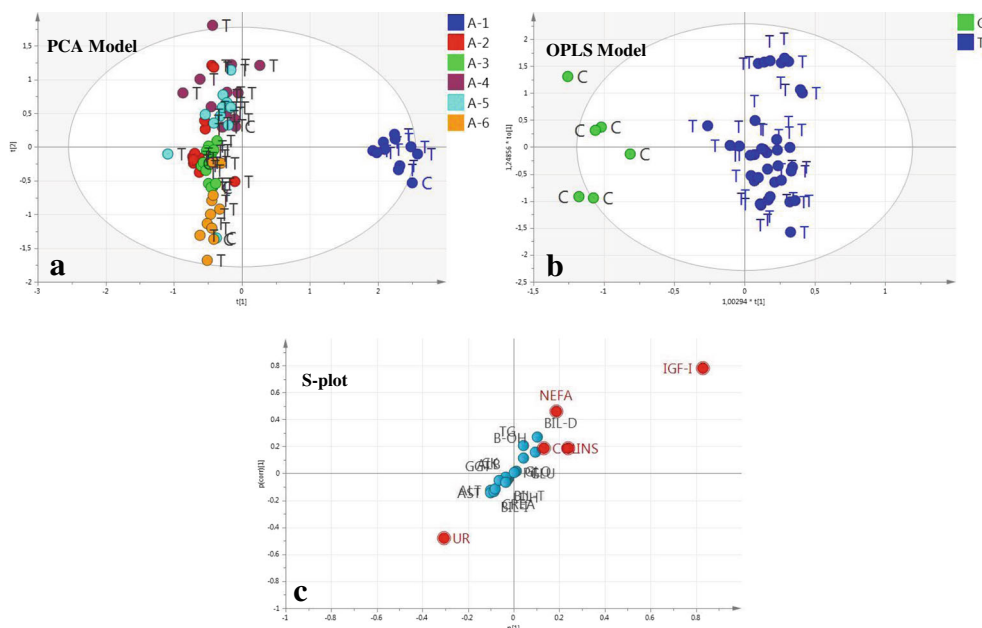
Before measuring plasma parameter concentrations in the samples of interest, the performances of the methods were determined and are reported in Table 1. As indicated, for all the targeted blood parameters, inter- and intra-assay variations never exceeded 10 %. These low variations were necessary requirements for the study and, in particular, for the subsequent statistical processing.

### Discrimination between treated and untreated bovines by multivariate statistical approaches

Firstly, a PCA analysis was applied for unsupervised purposes and assessment of the general variance associated to blood parameter concentration. A PCA model representing the linear combinations of the 21 targeted analytes measured from experiment A (Fig. 1a) resulted in the following model characteristics:  $R^2(X_1)=0.37$ ,  $R^2(X_2)=0.18$  and  $Q^2=0.32$  (where  $R^2$  expresses the model's descriptive capacity and  $Q^2$  defines the model's predictive power). No analytical bias resulting from the analysis order was observed, and no outlier could be highlighted; data were then considered as relevant to describe only biological variability. From the score scatter plot of the PCA model, the model mainly explained the variability arising from the differences between animals than related to treatment; nevertheless, a slight discrimination could be observed between the samples collected before treatment and those collected between D2 and D9.

In order to discriminate the samples ( $X$  variable) based on their respective status ( $Y$  variable, i.e. control or treated), the supervised OPLS was carried out. According to previous observations, the OPLS model was built based on the data set obtained from the samples collected on D0 and D2 to D9, which resulted in the score scatter plot presented in Fig. 1b. The characteristics of the OPLS model were  $R^2X=0.64$ ,  $R^2Y=0.91$  and  $Q^2=0.76$ . The model succeeded in ensuring a distinct separation between the two sample classes with a high statistical relevance ( $P=2.10^{-9}$ ). In order to determine the most relevant variables in this discrimination, the corresponding S plot was used (Fig. 1c). On such figure, the analytes are plotted according to their contribution to the predictive component ( $p[1]$  axis) associated to their confidence level ( $p_{\text{corr}}[1]$  axis).

**Fig. 1** Multivariate statistical analyses for experiment A based on the 21 targeted blood parameters. Score scatter plot of the PCA model (a), score scatter plot of the OPLS model (b) and associated S plot (c, the most discriminating blood parameters are highlighted in red) (days 0 vs. 2 to 9 ( $n=42$ )) for experiment A. C corresponds to the samples from control animals (i.e. samples collected before treatment), and T corresponds to the samples from treated animals (i.e. samples collected after treatment)



Thus, the signals located at both ends of the S plot correspond to the variables mainly involved in the discrimination. The blood parameters plotted on the left side of the model correspond to those with depleted concentrations in the plasma after rbST administration, while those plotted on the up-right side correspond to the analytes with a significantly higher level in the plasma upon treatment. The S plot showed that IGF-I, urea (UR), NEFA, insulin (INS) and cholesterol (COL) were the most discriminating analytes. As expected, IGF-I was observed as the most impacted parameter upon rbST administration. Indeed, IGF-I is known to be concentration dependent with somatotrophin and therefore was already reported in several studies as a biomarker of rbST abuse [4]. Thus, a new OPLS model was built only with the five selected blood parameters (namely, IGF-I, UR, NEFA, INS and COL), and the discrimination between the control samples and treated samples from days 2 to 9 was still statistically relevant ( $P=0.001$ ).

Then, the final OPLS model was statistically evaluated with a permutation test (number of permutations=50) and the cross-validation step consisting in building a new model with two thirds of the randomly selected samples from experiment A and predicting the plotting of one third of the remaining samples on the newly established model was carried out [16]. The correct prediction of one third of the samples ensured the statistical robustness of the model.

The next step consisted in robustness evaluation of the model with supplementary samples from untreated bovines; therefore, several samples from cows which had never been treated with rbST (control 1 and control 2, see Table 2) were predicted on the model. These control samples were collected from different animals ( $n=6$ ) of different ages (3 and 4 years old) and over several days at different points (morning or afternoon). As expected, the OPLS model, based on only five

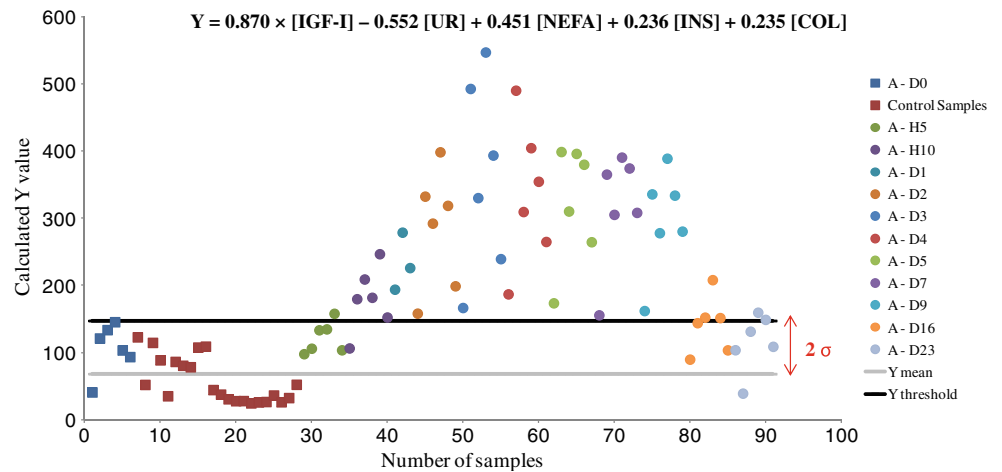
variables, allowed the correct classification of almost all the control samples (only two samples were not correctly predicted). No effect of ages, sampling days or hours could be observed, which confirms that combining several parameters in a predictive model minimizes the influence of biological intra- and inter-variability.

Status prediction of samples using a simple mathematically weighted equation

Considering both statistical relevance and robustness of the developed model, the individual contribution of each biomarker was extracted from the S plot in order to obtain a simple mathematically weighted equation allowing status prediction of samples. Individual contributions were as follows: IGF-I (+0.870), UR (-0.552), NEFA (+0.451), INS (+0.236) and COL (+0.235), leading to the following weighted equation:  $Y=0.870 \times [\text{IGF-I}] - 0.552 \times [\text{UR}] + 0.451 \times [\text{NEFA}] + 0.236 \times [\text{INS}] + 0.235 \times [\text{COL}]$ . Based on individually measured concentrations, this equation determined the coordinates of a given sample on the model.

$Y$  values for the control samples from experiment A (A-D0) and for the 22 control samples (control 1 and control 2) resulted in a mean  $Y$  value ( $Y_m$ ) of 68.02 (standard deviation (SD) 39.55) for control samples. In order to set a criterion allowing the prediction of suspicious rbST-treated samples, a  $Y$  threshold ( $Y_t$ ) was determined as the  $Y$  mean value ( $Y_m$ ) added to twice the SD (corresponding to the 95th percentile level of confidence) and was set at 147.12. Finally,  $Y$  values for the treated samples from experiment A were calculated as indicated in Fig. 2. From days 1 to 9, all the samples presented  $Y$  values above the threshold. Efficiency of the threshold was proven over the period days 1–9 after treatment, while before

**Fig. 2** Graphical representation of *Y* calculated values for experiment A and control samples (A-D0; control 1 and control 2). The *Y* threshold was set in order to predict the sample status (*Y* values, above this threshold, were declared as suspicious)



and after, performances were less efficient (46 % of the samples presented *Y* values above the *Y* threshold). Thus, the developed weighted equation succeeded in predicting the treated samples from experiment A from days 1 to 9 with a false-negative rate of 0 %.

For comparison purposes and to confirm the interest of combining several markers in a diagnostic tool, the same approach was applied with only IGF-I used as biomarkers. Based on control samples (A-D0, control 1 and control 2), a threshold for IGF-I plasmatic concentration could be established at 160.5 ng mL<sup>-1</sup>. In this case, the false-negative rate would be 15 % from days 1 to 9 after treatment, while before and after treatment, only 10 % of the samples were correctly classified.

**Preliminary assessment of the model**

The previously developed screening criterion, based on the five selected blood parameters, was applied on a large set of additional sample as follows: 20 supplementary control samples collected from 13 cows (B-D0; C-D0; control 3 and control 4) and 42 treated samples from experiments B and C (B

and C). In the same time, IGF-I values of these samples were compared to the IGF-I threshold set at 160.5 ng mL<sup>-1</sup>. The results in terms of false-negative and false-positive rates are presented in Table 3. As indicated, the developed screening method based on the five selected blood parameters succeeded in predicting the compliance status of the supplementary control samples with a false-positive rate of only 10 %. This result was more than satisfactory as the control samples came from different animals of different ages, resulting in a high biological variability. Concerning the prediction of additional samples from rbST-treated animals (experiments B and C), almost all the samples from days 1 to 9 after the last injection of rbST were correctly classified, resulting in a false-negative rate of only 4.5 % in accordance with EU requirements for screening methods (Decision 2002/657/EC). For samples collected before day 1 and after day 9, the results in terms of false-negative percentage were 66 and 79 %, respectively. These results indicated that physiological disorders of blood parameters happened after the first day of injection until day 9. After day 9, initial metabolomic levels of the selected analytes were restored. Compared to the results obtained with IGF-I-based model, both false-positive and false-negative rates were

**Table 3** Results in terms of false-positive (FP) and false-negative (FN) rates for the proposed screening criteria. Results are based on (i) only IGF-I values and (ii) *Y* values calculated from the five selected blood

parameters for the supplementary control samples (*n*=20) and for experiments B and C (*n*=42) and compared to corresponding thresholds

Samples	Control	Samples from treated animals			
		Experiments B and C (just after treatment and before day 1)	D1 to D9, <i>n</i> =22		Experiments B and C (after D9 or after D23)
			Experiments B and C (between D1 and D9)	Experiment B (between D15 and D23 after a second injection at D14)	
<i>N</i>	20	6	16	6	14
IGF-I only	% FP=15	% FN=83	% FN=25 % FN=18	% FN=0	% FN=86
5 blood parameters model	% FP=10	% FN=66	% FN=6 % FN=4.5	% FN=0	% FN=79

improved thanks to the combination of several blood parameters. To conclude, the preliminary assessment of the proposed screening criterion, based on only five blood parameters, was very satisfactory regarding its ability to predict rbST-treated cows.

## Conclusions

This study showed that rbST treatment induced disorders in blood parameter concentration through the profiling of 21 targeted analytes. The 21 targeted clinical analytes, routinely measured in veterinary practices as representative of the physiological, nutritional and metabolic status of farm animals, together in a statistical model, have showed their relevance to discriminate samples from treated and untreated animals. The dedicated and efficient statistical analyses permitted to highlight five potential biomarkers (IGF-I, UR, NEFA, INS and COL) of rbST administration. Taking into account their respective contributions, a mathematically weighted equation was proposed and a threshold was set. Statistical robustness of the model was validated, and the first evaluation of the proposed screening criterion was encouraging with regard to its ability to deal with biological inter- and intra-variations (age, season, physiological status, etc.). Additional parameters such as sampling time point in relation with feeding of the animal should be considered in a next step, especially with regard to INS levels. Furthermore, since a long-term treatment might lead to altered biomarker responses, issues relating to chronic treatment with rbST will also have to be assessed in the near future as part of the validation of the model and before routine application. Moreover, the complete validation of this biomarker-based approach requires more samples both from control and treated animals; this will be undertaken in the near future.

To conclude, this study showed that the combination of only five classical blood parameters may be used as a potential screening tool to detect rbST abuse in lactating cows.

Currently, the screening strategy for rbST misuse is based on the detection of antibodies raised against rbST, which is only possible more than a week after treatment and thus does not cover the early period. The proposed criterion allows covering an early period (from days 1 to 9), and its implementation together with the current screening method would allow more efficient screening of an rbST treatment since covering complementary detection windows. Moreover, the overlay of the detection window of the proposed screening approach and current confirmatory method (based on the detection of rbST in the blood only possible during 4 days after the last injection of rbST) makes the proposed approach particularly relevant.

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