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Toxicogenomic markers for corticosteroid treatment in beef cattle: Integrated analysis of transcriptomic data.

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Abstract

In the present work, an integrated analysis was performed on DNA-microarray data of bovine muscle samples belonging to controls, animals treated with various growth promoters (GPs) and unknown commercial samples. The aim was identify a robust gene expression signature of corticosteroid treatment for the classification of commercial samples, despite the effects of biological variation and other confounding factors.

DNA-Microarray data from 5 different batches of bovine skeletal muscle samples were analyzed (146 samples). After preprocessing, expression data from animals treated with corticosteroids and controls from the different batches (89 samples) were used to train a Support Vector Machines (SVMs) classifier. The optimal number of gene probes chosen by our classification framework was 73. The SVMs with linear kernel built on these 73 biomarker genes was predicted to perform on novel samples with a high classification accuracy (Matthew's correlation coefficient equal to 0.77) and an average percentage of false positive and false negative equal to 5% and 6%, respectively.

Concluding, a relatively small set of genes was able to discriminate between controls and corticosteroid-treated animals, despite different breeds, animal ages, and combination of GPs. The results are extremely promising, suggesting that integrated analysis provides robust transcriptomic signatures for GP abuse.

Keywords

- Integrated analysis;
- DNA-microarray;
- Bovine;
- Muscle;
- Biomarker

1. Introduction

The European Union has long established a strong policy against the use of growth promoters (GPs) in animal husbandry, to guarantee food safety, animal welfare, and consumer health. The use of hormonal compounds has been banned since 1988 and more recently directive 96/22/EC maintained the prohibition on such substances for growth promoting purposes, extended the ban to beta-agonists; restricted the use of hormones for therapeutic or zoo-technical purposes, and reinforced controls. Directive 96/23/EC also defines standards for official tests. Controls should be performed by routine or field laboratories (RFLs) coordinated and controlled by at least one national reference laboratory (NRL), designated by the national government. Each EU member state is required to adopt a National Monitoring Plan to survey the presence of residues in live animals, their tissues and fluids, and animal products. Finally, based on the Opinion of the Scientific Committee on Veterinary Measures, 1999, Opinion of the Scientific Committee on Veterinary Measures, 2000 and Opinion of the Scientific Committee on Veterinary Measures, 2002, the European Commission adopted Directive 2003/74/EC, which amends Directive 96/22/EC in relation to the permanent prohibition for the use of hormones in stock farming.

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Despite controls, however, anabolic compounds are still administered to increase growth performances and feed conversion rate. With regard to the illegal use of corticosteroids in beef cattle, results of the Italian Monitoring Plan (PNR) reported 22 non-compliant cases on 3332 (0.7%) analyzed samples for PNR 2011 and 32 on 3694 (0.9%) for PNR 2012. The real impact of illegal use of GPs, however, might be underestimated because various strategies are adopted to elude official controls, such as the administration of different compounds with similar biological action each at very low dose, the use of different classes of compounds with additive or synergic effects at low doses (*e.g.* corticosteroids and beta-agonists); the development of new chemical species, the use of natural steroids or their precursors, which might be confounded with those of endogenous origin (Courtheyn et al., 2002).

The extremely articulate scenario described above highlights the need to support the existing methods with new analytical approaches to identify putative risks for the consumer's health but also to increase the knowledge of metabolic patterns and associated kinetics of elimination for GPs.

For live animals, animal products and their feed, the Council Directive 96/23/EC still considers only the parental compound or its metabolite as proof for the administration of illegal compounds detected by confirmative methods based on mass spectrometry. Some steps forward have been done for anti-doping enforcement in humans and horses since the presence of markers or scientific indicators is considered a sufficient and officially accepted proof for doping, according to the WADA's Executive Committee, (WADA's Athlete Biological Passport Operating Guidelines, ver-4.0 2013) to the International Federation of Horseracing Authorities (International Agreement on Breeding, Racing and Wagering, April 2014), respectively.

With respect to indirect biomarkers, histological techniques and analysis of blood chemistry parameters have long been proposed and in some cases officially adopted as alternative screening methods in prevention of GP abuse. The advent of "omics" technologies (genomics, transcriptomics, proteomics, metabolomics) now offers interesting perspectives to discover reliable and quantifiable markers, which however still await for full implementation in routine analysis. Transcriptome analysis using DNA-microarrays has been recently explored as a screening method to reveal the biological effects of different anabolic compounds and thus support the existing tools to combat the use of these substances in beef cattle (Cannizzo et al, 2013, Carraro et al, 2009, De Jager et al, 2011, Pegolo et al, 2012 and Rijk et al, 2010).

In the detection of anabolic treatments, some advantages of gene expression profiling with respect to the existing conventional methods have already been discussed (Pegolo et al., 2012). For instance, a trascriptomic approach allowed to identify the effects of GPs administration even when the parental compound or its metabolites were no longer detectable in biological fluids by LC-MS/MS methods or in absence of histological alterations (Cannizzo et al., 2013).

Despite many advantages, gene expression biomarkers proposed to detect animals illegally treated (e.g. Divari et al, 2011, Giantin et al, 2010 and Riedmaier et al, 2011) might be influenced by intrinsic or extrinsic factors (*e.g.* age, breed, diet). Besides, identification of potential biomarkers is carried out in controlled experiments, which often use few animals and consider a small number of factors/variables. As a result, high throughput analyses carried out in different laboratories often give different biomarker lists and different features may be selected under different settings. This is a general problem that has been encountered in the application of gene expression markers in other fields, where possible solutions have been proposed (Abeel et al, 2010, Di Camillo et al, 2012 and Sanavia et al, 2012). In particular, it has been suggested to increase the number of cases

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through meta-analysis or integrated analysis of results from different studies, while at the same time implementing rigorous statistical methods to account for data heterogeneity.

In the present work, an integrated analysis was performed on DNA-microarray data of bovine muscle samples belonging to controls, animals treated with various combination of GPs and also unknown commercial samples; in particular, a Support Vector Machines (SVMs) classifier was trained on data from animals treated with corticosteroids and controls (89 samples). Gene expression profiles for the majority of samples were already presented in previous studies. However, microarray data for two additional groups of samples were produced specifically for the present paper. The first group included younger animals (treated and controls) while the second group consisted of muscle samples preserved under vacuum at 4 °C for 14 days, to mimic meat storage under commercial conditions and to test RNA stability. In fact, the ultimate goal of this study is to explore the feasibility of using gene expression markers as a routine analytical tool on commercial samples, through the identification of a gene expression signature for corticosteroid treatment that is robust despite the effects of biological variation and other confounding factors, but also assessing the possibility to work along the whole supply chain by analyzing samples long after slaughtering.

2. Material and methods

2.1. Sample batches

DNA-Microarray data from 5 different batches of bovine skeletal muscle samples were analyzed for a total of 146 samples. Of these samples, 51 correspond to animals treated with corticosteroids, 32 were treated with different combination of GPs, 38 correspond to control animals, 25 are of unknown class. The 89 samples corresponding to animals treated with corticosteroids (51 samples) and controls (32 samples) were used here to train a Support Vector Machines classifier. The others were nevertheless included in the microarray data preprocessing steps to render more robust the estimate of ComBat and quantile normalization parameters, useful to combine data from different batches (as explained in section 2.5).

Batch 1 includes data from *biceps brachii* (*bb*) samples (45) of male Holstein cows, 12–25 months old, 450–700 kg in body weight. Group F (n = 8) animals were chronically exposed for 92 days to a steroid hormone implant containing 200 mg trenbolone (Finaplix-H). Group R (n = 8) animals were chronically exposed for 89 days to a steroid hormone implant containing 200 mg trenbolone and 20 mg E (Revalor-200, Intervet, USA). Group RD (n = 8) animals were chronically exposed (89 days) to a Revalor-200 implant and were also orally administered with dexamethasone (DEX; 0.7 mg/day/animal) from day 45 to day 84. Group C (n = 13) consisted of untreated animals and served as control ((Pegolo et al., 2014). Group P (n = 8) animals were orally administered with prednisolone acetate (Novosterol, Ceva Vetem spa, Italy) 30 mg day^{-1} for 35 days (Cannizzo et al., 2013). Microarray data were deposited in NCBI Gene Expression Omnibus (Edgar et al., 2002) and accessible through GEO Series accession numbers GSE47827 and GSE50036.

Batch 2 is composed by microarray data from *bb* samples (16) of male mixed breed (Charolaise X Limousine) cows, 18 months old, 450 kg body weight. Animals were divided into three groups: five were untreated (controls); five were administered with DEX via feed 0.75 mg/head daily for 43 days (group DEX); the last six animals were administered via feed for 43 days with DEX (0.75 mg/head) and intramuscularly three times with E, 20 mg/head (group DEX+E) after 7 days, 21

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days, and 35 days from the start of the experiment (Carraro et al., 2009). Microarray data are accessible through GEO Series accession number GSE12179.

Batch 3 is represented by microarray data from *bb* of unknown commercial samples (25) of male Charolaise, Limousine and mixed breed cows, 12–24 months old, weight between 300–500, and accessible through GEO Series accession number GSE26318.

Batch 4 embraces *bb* muscle samples (40) from male Holstein calves, 6–7 months old, weight between 200–290 kg experimentally treated with different combination of GPs. Group P (n = 8) animals were orally treated with prednisolone acetate (15 mg/animal/day) for 31 days; Group BE (n = 8) animals were intramuscularly weekly treated with estradiol benzoate (5 mg/animal) for six weeks plus brotizolam *per os* (0.02 mg/Kg/day) for 31 days; Group DE (n = 6) animals were intramuscularly weekly treated with estradiol benzoate (5 mg/animal) for six weeks plus DEX *per os* (0.4 mg/animal) for 31 days; Group RN (n = 8) animals were orally treated with ractopamine (80 mg/animal) for 31 days plus 4 intramuscular injection of nandrosol (150 mg/animal) every 2 weeks; Group C (n = 10) consisted of untreated animals and served as control (Fig. 1).

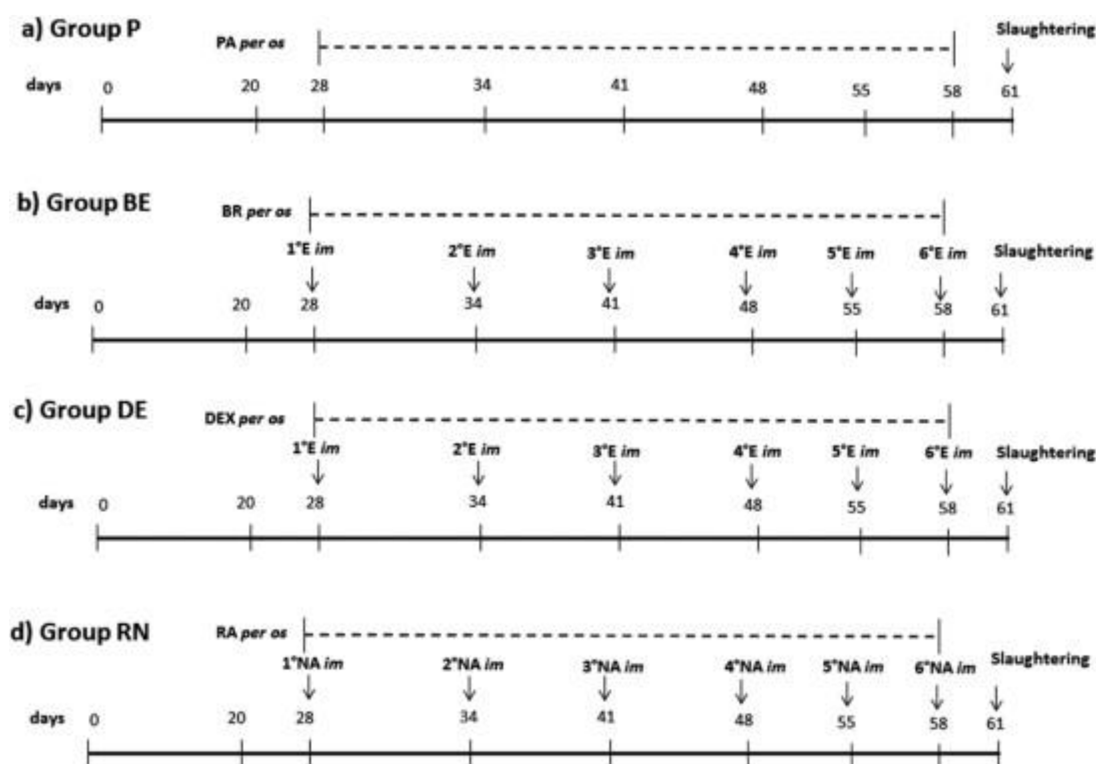

Fig. 1.

Treatment protocols for batch 4. a) Group P. Animals treated with prednisolone acetate (PA, 15 mg/animal/day) *per os* for 31 days; b) Group BE. Animals weekly intramuscularly treated with estradiol benzoate (E, 5 mg/animal) for six weeks plus brotizolam (BR) *per os* (0.02 mg/Kg/day) for 31 days; c) Group DE. Animals weekly intramuscularly treated with estradiol benzoate (E, 5 mg/animal) for six weeks plus Dex *per os* (0.4 mg/animal) for 31 days; d) Group RN. Animals treated with ractopamine (RA, 80 mg/animal) *per os* for 31 days plus 4 intramuscular administrations of nandrosol (NA, 150 mg/animal) every 2 weeks. Group C was used as controls. All animals were slaughtered three days after drug withdrawal.

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For oral administration, before the distribution of feed each morning, animals were tied to the feeding trough, where two trained technicians gave one capsule containing the compound per application, using a drenching gun.

All groups of experimental animals were kept in separate boxes, 10 m × 10 m, and tethered and fed with liquid milk replacer, twice a day [crude protein 22.50%, crude oil and fats 22.50%, crude fiber 0.30%, crude ash 6%, calcium 0.65%, sodium 0.5%, phosphorus 0.65%, cellulose 0.001; vitamin A 25.000 IU/kg, vitamin C 50 mg/kg, Cu 5 mg/kg, vitamin D3 3.700 IU/kg, vitamin E 75 mg/kg]. The amount of the fodder was increased gradually up to 16 l/calf/day; after one month 0.5 kg of barley straw (D.M. 0.90, C.P.0.02, E.E. 0.01, ash 0.06, crude fiber 0.41) was added to the diet according to the indications suggested by the European Commission (97/182/EC). Carcasses of treated animals were destroyed (2003/74/CE – DL 16 March 2006, n. 158).

The experiment was run in an authorized facility according to the European Community Directive 86/609, recognized and adopted by the Italian Government (DLgs 116/92). The experimental plan was approved by the Italian Ministry of Health.

Batch 5 is composed of neck muscle samples (20) from the DE (5 samples) and the C (5 samples) groups of Batch 4 either stored as for all other samples in RNAlater (Batch 5, samples D0) or kept under vacuum at 4 °C for 14 days and subsequently placed in RNAlater and stored at −20 °C as all other samples (Batch 5, samples D14).

2.2. RNA extraction

Total RNA was extracted from *bb* skeletal muscle samples stored in RNAlater at −20° animals using the RNAeasy Mini kit (Qiagen, Hilden, Germany).

The concentration of RNA samples was measured using a UV-vis spectrophotometer NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE) and RNA integrity was estimated running each sample on a RNA-chip in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA integrity number (RIN) was evaluated using the Agilent 2100 Expert software and used as a measure to standardize the interpretation of RNA quality, which is no longer determined by the ratio of ribosomal bands, but by the entire electrophoretic trace of the RNA sample.

2.3. RNA amplification, labeling and hybridization

DNA-microarray experiments were conducted on samples from Batch 4 and 5, the other data being available from previous studies. Sample labeling and hybridization were performed according to the Agilent Low Input Quick Amp Labeling protocol. Briefly, for each sample 100 ng of total RNA were linearly amplified and labeled with Cy3-dCTP. A mixture of 10 different viral poly-adenylated RNAs (Agilent Spike-In Mix) was added to each RNA sample before amplification and labeling, to monitor microarray analysis work-flow. Labeled cRNA was purified with Qiagen RNAeasy Mini Kit, and sample concentration and specific activity (pmol Cy3/μg cRNA) were measured in a NanoDrop ND-1000 spectrophotometer. A total of 1650 ng of labeled cRNA was prepared for fragmentation adding 11 μL 10 × Blocking Agent and 2.2 μL of 25 × Fragmentation

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Buffer, heated at 60 °C for 30 min, and finally diluted by addition with 55 µL 2 × GE Hybridization buffer. A volume of 100 µL of hybridization solution was then dispensed in the gasket slide and assembled to the microarray slide (each slide containing four arrays). Bovine-specific oligo-arrays (Agilent Bovine-Four-Plex G2519F) were used. For most of the transcripts represented on this array, two identical probes are synthesized at two distinct positions on the slide, therefore the average value between the intensities of the two replicate probes was used. The slides were incubated for 17 h at 65 °C in an Agilent Hybridization Oven, subsequently removed from the hybridization chamber, quickly submerged in GE Wash Buffer 1 to disassembly the slides and then washed in GE Wash Buffer 1 for approximately 1 minute followed by one additional wash in pre-warmed (37 °C) GE Wash Buffer 2. Hybridized slides were scanned at 5 µm resolution using an Agilent G2565BA DNA microarray scanner. Default settings were modified to scan the same slide twice at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). Microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession numbers GSE52779 and GSE52883 for Batch 4 and Batch 5, respectively.

2.4. Microarray data quality control and pre-processing

Agilent quality reports of each array were analyzed to assess the overall reproducibility of single replicate in the experimental design and to make a decision on filtering genes or entire arrays from an experiment. In addition, the MvA plots, representing gene expression differences between arrays (M) against their average (A) in log scale, were examined to further assess sample variability and possible signal artifacts at different average expression intensities.

All data were log transformed (base 2) and signals from different probes mapping on the same genes were averaged.

Batches 1, 2, 4 and 5 were resampled in B = 100 training/test samples, as explained in the following paragraph. In turn, samples from different batches belonging to the train were combined using ComBat, an empirical Bayesian framework that adjusts data for batch effects and is robust to outliers in small datasets (Johnson et al., 2007). ComBat was used in its parametric mode, defining batches based on cattle cohorts. Finally, data training data were normalized using quantile normalization.

Batch 3, which consists of samples collected at commercial slaughterhouses by unskilled staff and previously analyzed and classified with other approaches (Pegolo et al., 2012), was not used either as training or test for feature selection and classification (for which only batches 1, 2, 4 and 5 were used), but was used, at the end of the learning process as an external, previously unseen validation set.

2.5. Feature selection and classification

After preprocessing, data from animals treated with corticosteroids and controls belonging to batch 1, 2, 4 and 5 (89 samples) were used to train a Support Vector Machines (SVMs) classifier (Vapnik, 1998). SVMs are a set of supervised learning methods used for classification, in principle able to identify nonlinear features thus providing a more complete set of relevant genes. They were used here with linear (LSVM) and Gaussian kernel (GSVM).

The Matthews correlation coefficient, MCC (Baldi et al., 2000), was used as a measure of classification accuracy and was calculated directly from the confusion matrix by the formula:

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$$MCC = \frac{TP \cdot TN - FP \cdot FN}{\sqrt{(TP+FP)(TP+FN)(TN+FP)(TN+FN)}}$$

where, TP is the number of true positive, TN the number of true negative, FP the number of false positive and FN the number of false negative subjects.

SVMs are used in a Monte Carlo bootstrap resampling scheme with $B = 100$ external training/test splits (Fig. 2, left panel). This is an approach broadly detailed and used in the literature (Ambroise, McLachlan, 2002, Di Camillo et al, 2012 and Furlanello et al, 2005).

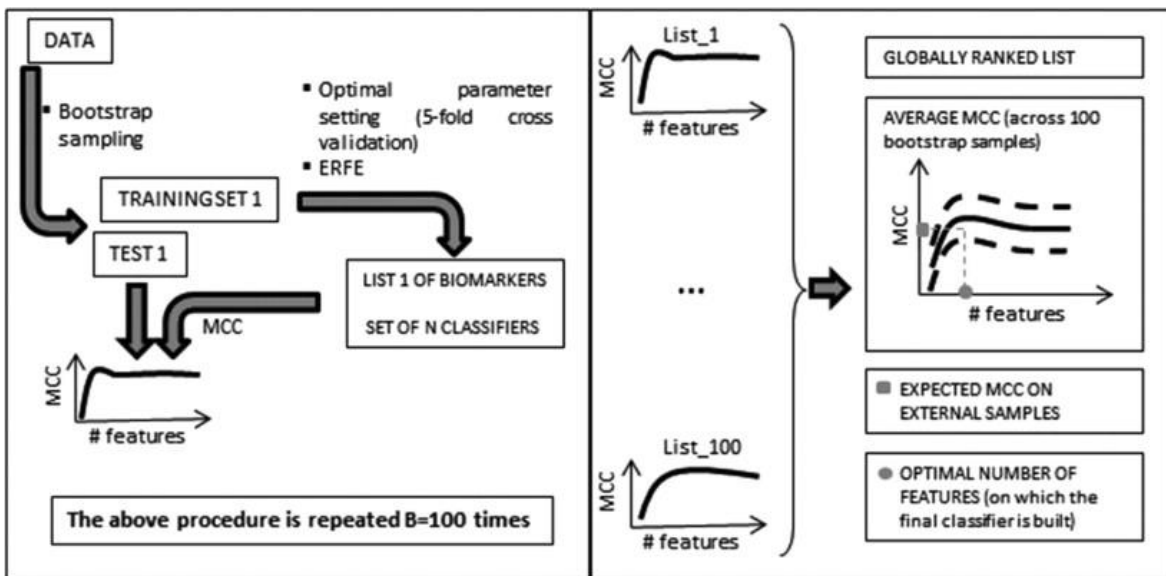

Fig. 2.

SVMs bootstrap resampling scheme adopted for learning. Left Panel: The Entropy-based Recursive Feature Elimination (ERFE) procedure was used as the ranking scheme within each bootstrap sample. Optimal parameter setting was performed through a 5-fold cross-validation within each Monte Carlo classifier. For each of the B = 100 Bootstrap samples, the analysis pipeline gave as output: i) a list of biomarkers ranked based on their ability to classify the samples in the training; ii) a set of N classifiers obtained using the first n ranked features (n = 1,2, …, N). Right Panel: The MCCs of these classifiers were evaluated for each of the B = 100 Bootstrap samples, on B external test samples across B lists of ranked features. These lists were merged in a globally ranked list and the average MCC across the 100 Bootstrap samples was calculated for different numbers of features. Finally, the optimal number of features on the globally ranked list (red dot) was chosen correspondingly to the optimal MCC (red square).

In detail, for each run b ($b = 1, \ldots, B$), $N = 89$ examples (i.e. bovine samples) are sampled with replacement from the original 89 to build the training set, meaning that, at each run, some examples

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can be sampled more than once and others are not selected as training examples. These latter examples thus form the test set for run *b*.

The training set then undergoes a more classical 5-fold cross-validation to optimally tune the value of the regularization parameter *c* (the trade-off between empirical error and smoothness of the solution) and, for the Gaussian kernel, of the bandwidth σ. The parameter choice is based on the optimal value of MCC, which is estimated through a 5-fold cross-validation, internal to each Monte Carlo classifier. Within each run *b*, the Entropy-based Recursive Feature Elimination (ERFE) procedure is then used to rank variables (Furlanello et al., 2003). Starting from the classical RFE algorithm (Guyon et al., 2002), ERFE adaptively discards a subset of the least informative features according to an entropy measure of the distribution of the weights generated by the feature weighting schema. The last 100 features were ranked using classical RFE. Thus, for each of the B = 100 Bootstrap samples, the adopted analysis pipeline gives as output: (i) a list of biomarkers ranked based on their ability to classify the samples in the training; (ii) a set of N classifiers obtained using the first n ranked features (n = 1,2, …, N). Only at this point the test set *b* is used to give an unbiased estimate of the *MCC*, which is calculated for the set of the N different classifiers obtained using the first n ranked features.

The entire procedure is repeated *B* = 100 times. The *B* = 100 resulting ranked lists of biomarkers finally merged in a globally ranked list using the average rank of each feature. Correspondingly, the average MCC across the 100 Bootstrap samples is calculated for different numbers of features. Finally, the optimal number of features on the globally ranked list is chosen correspondingly to the optimal MCC (Fig. 2, right panel).

The final classifier was built on these features using the entire dataset. As stated above, its expected MCC (Eq. 1) on external, previously unseen samples is obtained by averaging the MCCs obtained on test samples across the *B* = 100 bootstrap runs.

This strategy, i.e. applying the bootstrap externally to the selection process, has been proved to be an effective countermeasure against unwanted selection bias effects able to (i) realistically estimate the classification accuracy on external, previously unseen samples (Ambroise and McLachlan, 2002); (ii) select biomarkers in a precise and reproducible way (Di Camillo et al, 2012 and Furlanello et al, 2005).

It must be pointed out that the training data in each bootstrap sample undergo the preprocessing at each run; parameters of ComBat and quantile normalization are saved and then used to normalize the test set. In this way, the test sets are entirely analyzed as new groups of samples coming to the analysis for the first time and thus provide an accurate estimate of the performance of the classifier on new, previously unseen samples.

All the analysis steps described in this and in the previous paragraph were implemented in R software (http://www.r-project.org/).

3. Results

In DNA microarray experiments, data quality is crucial. RNA integrity number (RIN) was used as a measure to standardize the interpretation of RNA quality. In the present study, a conservative threshold was enforced to reduce experimental biases due to poor RNA quality. For newly analyzed batches (batch 4 and 5), only RNA samples with RIN numbers ≥7 were further processed. The same

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criterion had been used for all other batches as already reported in previous papers. Based on the Agilent quality reports of each array, no sample was filtered out from the experiment. After microarray processing, MvA plots, representing gene expression differences between arrays (M) against their average (A) in log scale, were examined and samples 50 and 79 from batch 3 were excluded from further analysis because of their putative lower quality with respect to all other array data. Data were then log transformed (base 2) and signal from the two identical probes for the same transcript was averaged. The next step was the correction of batch effect, which represents a crucial passage when comparing a large set of microarray experiments. Batch correction was implemented in ComBat. Efficacy of batch correction can be appreciated comparing the distribution of samples along the first two dimensions after applying principal component analysis (PCA) on data before and after ComBat adjustment (Fig. 3).

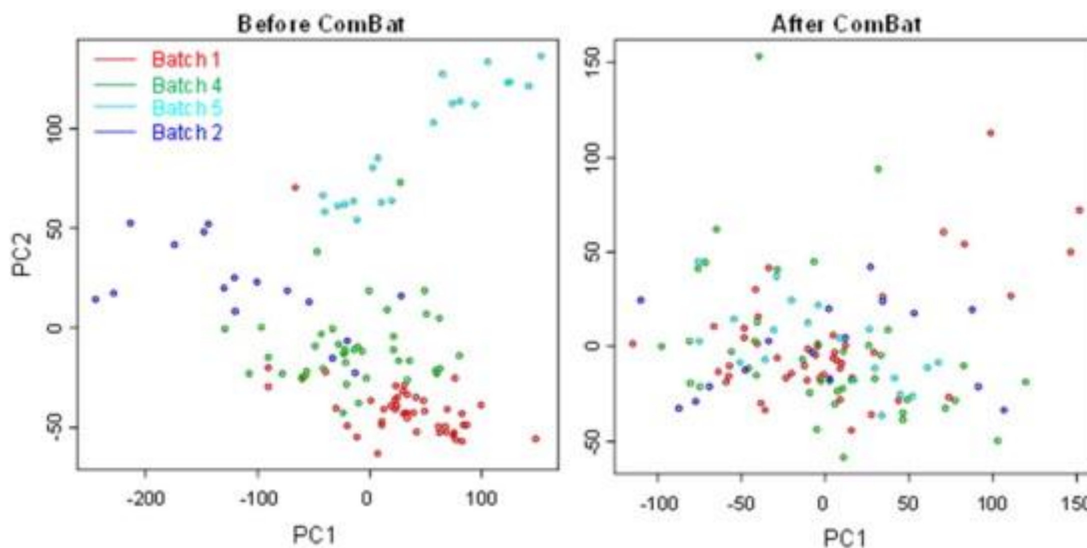

Fig. 3.

Principal component analyses for samples batches. The first and second principal components obtained applying PCA on microarray data before and after ComBat adjustment.

The last step was data normalization. A quantile normalization process was carried out to remove residual systematic differences between arrays and obtain data with the same distribution.

After pre-processing (quality filtering, batch correction, and normalization), a linear Support Vector Machine (LSVM) classifier and a Gaussian SVM (GSVM) one were trained on individual microarray data (20,602 genes per array) for a total of 89 muscle samples (controls and treated with corticosteroids). Microarray data for 45 samples (belonging to batch 1, 2) were produced in previous studies, while 44 samples (belonging to batch 4 and 5) were newly analyzed in the present work, using the same microarray platform and experimental protocols. The 57 residual samples belonging to different batches that are not controls or samples treated with corticosteroids were included only in the pre-processing (but not in the training phase) with the purpose of obtaining a more accurate estimate of the batch effect parameters with the package ComBat.

A summary diagram of sample batches used for the identification of the gene expression classifier is reported in Fig. 4.

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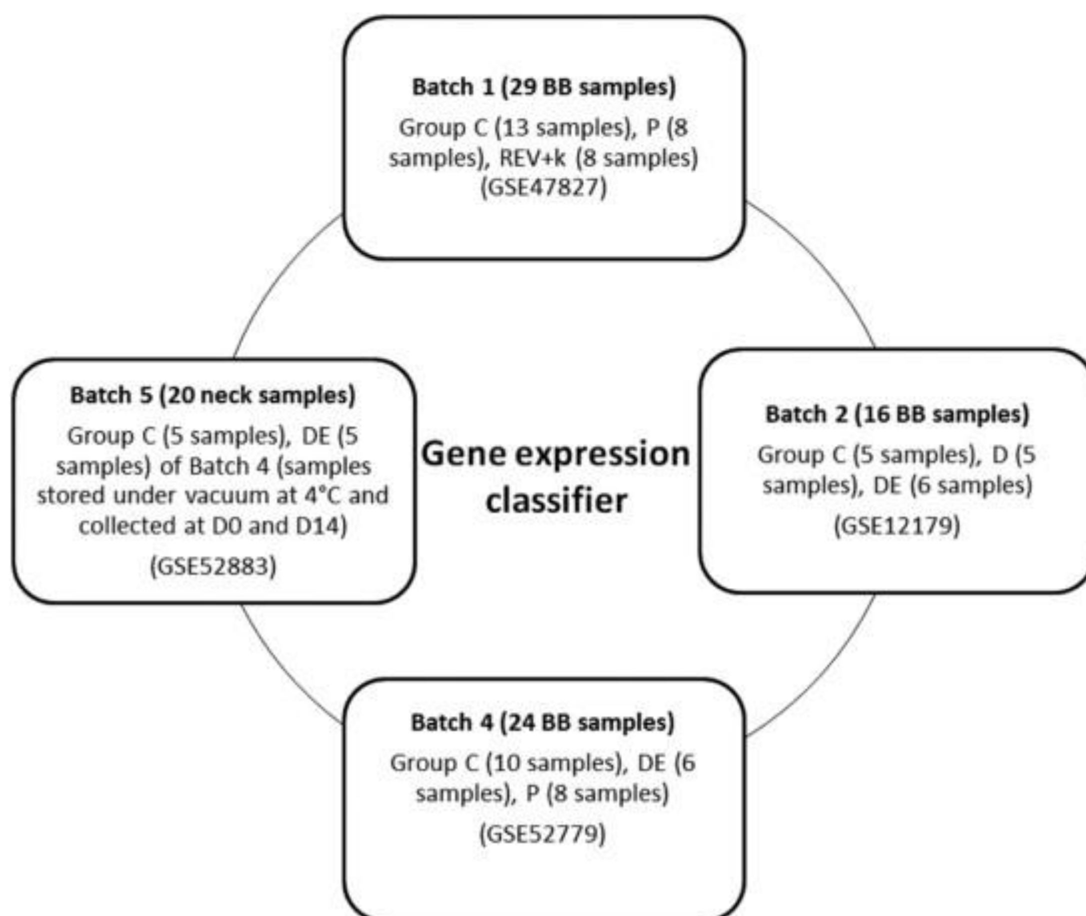

Fig. 4.

Summary diagram of sample batches used for the identification of the classifier. Batch 1: biceps brachii (*bb*) samples belonging to animals treated with Revalor plus Dex (group Rev+k), prednisolone (group P) and controls (group C); Batch 2: *bb* samples belonging to animals treated with Dex (group D), Dex plus estradiol (group DE) and controls (group C). Batch 4: *bb* samples from animals treated with Dex plus estradiol (group DE), prednisolone (group P) and controls (group C); Batch 5: neck samples belonging to group DE and group C of batch 4.

Samples were divided in two classes, the first one including animals treated with corticosteroids, either alone or in association with other compounds, the second one comprising control animals. As these two classes were numerically different, a specific measure of the quality of binary classification, namely, the Matthews correlation coefficient (MCC), was used. MCC provides a balanced measure of accuracy of classification, which can be used even if the classes are of different sizes. LSVM and GSVM performance, tested on the same dataset, gave an estimated MCC accuracy on external samples, *i.e.* samples not used for learning, equal to 0.77 and 0.63, respectively, thus showing that the linear kernel performs better than the Gaussian one. The optimal number of features (gene probes) chosen by our classification framework, which has been shown to allow selecting features with a high degree of precision and stability (Di Camillo et al., 2012), was 73.

Fig. 5 (left panel) shows the trend of the average MCC across the 100 bootstrap runs in dependency on the number of features (signal ± standard deviation in gray). The average MCC is quite stable for a number of features going from 15 to 80, with a maximum MCC in correspondence to 73 selected

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features. A more conservative choice would have been to select 15 features. Since, however, the objective of this work was to test all the required steps toward the identification of a robust and specific signature for corticosteroid treatment in bovine muscle samples, we preferred to consider the larger list, so as to assess in future studies the reproducibility of specific signals.

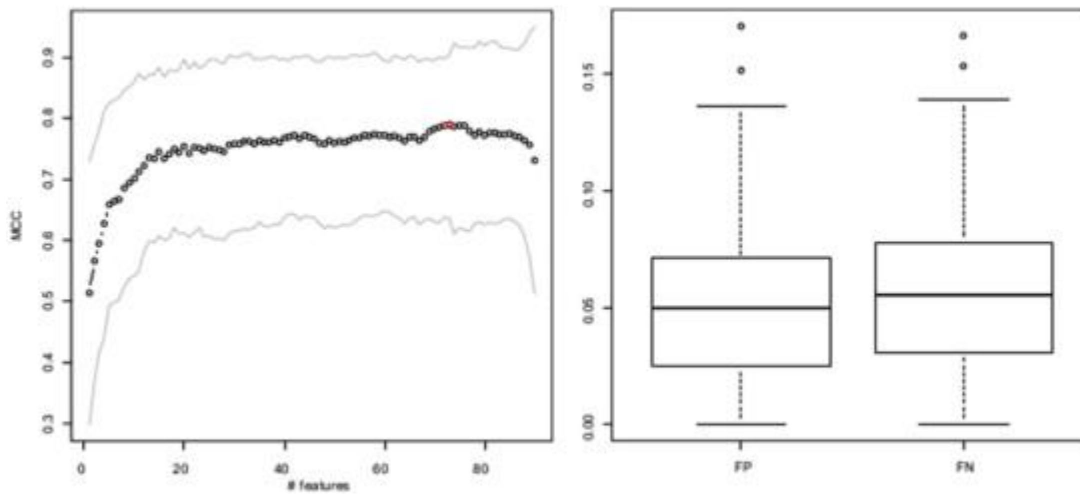

Fig. 5.

MCC, FP and FN distribution across the 100 bootstrap runs. Left panel: trend of the average MCC across the 100 bootstrap runs in dependency on the number of features (signal ± standard deviation in gray). Right panel: boxplots of the FP and FN classification errors across the 100 bootstrap runs when 73 features are used by the SVM classifier.

Figure options

The SVMs with linear kernel built on these 73 biomarker genes were thus used to build the final classifier (Table 1). This latter classified all the samples in the training set with 100% accuracy (0 classification errors; controls, animals treated with corticosteroids alone or in cocktail) and was predicted to perform on new, previously unseen samples with an average MCC accuracy equal to 0.77, as above stated, and an average percentage of FP (i.e. control samples classified as treated) and FN (i.e. treated samples classified as controls) equal to 5% and 6%, respectively. Fig. 5, right panel, shows the boxplots of the FP and FN classification errors across the 100 bootstrap runs when 73 features are used by the SVM classifier.

Table 1.

List of the 73 biomarker genes used to build the final classifier.

Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log 2FC	Ensembl transcript ID	Description	Functional categories
A_73_101163	XM_593659	FOXN4	2.50	2.93	0.43	ENSBTAT00000014397	Bos taurus forkhead box N4	Cell-cycle, cell proliferation, cell differentiation
A_73_10	NM_1741	PIM1	9.9	9.2	-	ENSBTAT0000	Bos taurus	

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log2FC	Ensembl transcript ID	Description	Functional categories
5828	44		1	3	0.68	0000511	pim-1 oncogene	
A_73_106276	NM_001192400	FGF6	2.02	2.36	0.34	ENSBTAT00000016675	Bos taurus fibroblast growth factor 6	
A_73_106673	NM_001045901	GADD45G	12.99	14.05	1.06	ENSBTAT00000003943	Bos taurus growth arrest and DNA-damage-inducible, gamma	
A_73_111205	NM_001191285	COL15A1	10.32	10.76	0.43	ENSBTAT00000013292	Bos taurus collagen, type XV, alpha 1	
A_73_114133	NM_001206261	RASD1	10.66	11.41	0.75	ENSBTAT00000027338	Bos taurus RAS, dexamethasone-induced 1	
A_73_115194	XM_866036	FAM3B	3.21	4.33	1.12	ENSBTAT00000043762	PREDICTED: Bos taurus similar to Protein FAM3B precursor (Cytokine-like protein 2–21), partial	
A_73_115755	XM_601664	SHCBP1	4.82	5.57	0.75	ENSBTAT00000047558	Bos taurus SHC SH2-domain binding protein 1	
A_73_116709	AV597868	RNF41	10.64	10.12	-0.52	ENSBTAT00000038204	Rep: Ring finger protein 41 – Bos taurus (Bovine), complete	

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log2FC	Ensembl transcript ID	Description	Functional categories
A_73_117986	XM_614662	ARRDC3	9.70	10.47	0.77	ENSBTAT00000009364	Bos taurus arrestin domain containing 3	
A_73_118155	NM_001034538	SNAI2	6.38	7.54	1.16	ENSBTAT00000017600	Bos taurus snail homolog 2 (Drosophila)	
A_73_119030	NM_001034247	GADD45A	10.89	11.57	0.68	ENSBTAT00000033450	Bos taurus growth arrest and DNA-damage-inducible, alpha	
A_73_121113	NM_001102276	RGCC	12.02	12.78	0.77	ENSBTAT00000049347	Bos taurus regulator of cell cycle	
A_73_105538	NM_001075144	ANPEP	5.15	5.74	0.59	ENSBTAT00000022456	Bos taurus alanyl (membrane) aminopeptidase	
A_73_100877	AW668786	MX1	4.78	5.28	0.50	ENSBTAT00000043742	Rep: Predicted protein – Monosiga brevicollis MX1, partial (9%)	Inflammatory-immune response
A_73_102188	CB437955	SMAD7	9.56	10.31	0.76	ENSBTAT00000046721	Bos taurus similar to Homo sapiens SMAD, mothers against DPP homolog 7 (Drosophila)	

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log2FC	Ensembl transcript ID	Description	Functional categories
A_73_102507	NM_001045868	NFKBIA	12.47	13.23	0.76	ENSBTAT00000022183	Bos taurus nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	
A_73_103623	AV591119	PTGIS	6.38	7.21	0.83	ENSBTAT00000023313	Rep: Prostacyclin synthase (EC 5.3.99.4) (Prostaglandin I2 synthase). – Xenopus tropicalis, partial (4%)	
A_73_106872	XM_616030	SMAD7	6.27	6.93	0.66	ENSBTAT00000046721	Bos taurus SMAD family member 7	
A_73_108457	NM_001080272	OSMR	9.33	8.61	-0.72	ENSBTAT00000061133	Bos taurus oncostatin M receptor	
A_73_110460	NM_001105340	TRAF5	4.24	5.00	0.76	ENSBTAT00000015940	Bos taurus TNF receptor-associated factor 5	
A_73_110993	NM_001078096	AOAH	4.68	5.15	0.47	ENSBTAT00000034676	Bos taurus acyloxyacyl hydrolase (neutrophil)	
A_73_111413	BM289321	RFX2	10.74	9.32	-1.42	ENSBTAT00000023492	Bos taurus regulatory factor X, 2 (influences	

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log2FC	Ensembl transcript ID	Description	Functional categories
							HLA class II expression)	
A_73_111433	NM_001076799	NOS2	7.96	8.40	0.45	ENSBTAT00000009062	Bos taurus nitric oxide synthase 2, inducible	
A_73_112955	NM_001199063	RGS1	7.53	9.48	1.96	ENSBTAT00000028880	Bos taurus regulator of G-protein signaling 1	
A_73_112988	NM_174006	CCL2	10.10	8.32	-1.78	ENSBTAT00000013146	Bos taurus chemokine (C-C motif) ligand 2	
A_73_117019	CB435429	IKZF1	2.76	3.59	0.83	ENSBTAT00000018632	Rep: IKAROS family zinc finger 2 – Bos taurus (Bovine), partial (11%)	
A_73_117872	NM_174184	SLAMF1	4.27	5.54	1.27	ENSBTAT00000010425	Bos taurus signaling lymphocytic activation molecule family member 1	
A_73_118454	NM_001144077	TNFRSF25	7.90	8.39	0.48	ENSBTAT00000008557	Bos taurus tumor necrosis factor receptor superfamily, member 25	
A_73_118788	XM_606449	C3	8.88	9.24	0.36	ENSBTAT00000022979	Bos taurus complement C3-like	
A_73_103432	XM_871133	PHLDA2	4.90	4.23	-0.67	ENSBTAT00000044171	Bos taurus pleckstrin homology-	Cell migration

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log2FC	Ensembl transcript ID	Description	Functional categories
							like domain, family A, member 2	
A_73_106754	NM_001098039	SH2D3C	7.80	8.64	0.85	ENSBTAT00000020433	Bos taurus SH2 domain containing 3C	
A_73_100440	NM_001081533	ZNF750	9.17	10.00	0.83	ENSBTAT00000034744	Bos taurus zinc finger protein 750	Regulation of transcription
A_73_105566	CB165926	ELL2	6.30	6.96	0.66	ENSBTAT00000009487	Bos taurus similar to Homo sapiens elongation factor, RNA polymerase II, 2	
A_73_118295	NM_001024929	BHLHE40	12.01	11.06	-0.95	ENSBTAT00000013008	Bos taurus basic helix-loop-helix family, member e40	
A_73_119473	NM_001076916	OSR1	8.63	9.35	0.72	ENSBTAT00000066336	Bos taurus odd-skipped related 1 (Drosophila)	
A_73_119969	CB457679	FZD7	10.72	10.11	-0.61	ENSBTAT00000002946	Bos taurus similar to Homo sapiens frizzled homolog 7 (Drosophila)	
A_73_118758	NM_174147	PLAU	11.09	10.30	-0.79	ENSBTAT00000007806	Bos taurus plasminogen activator, urokinase	Wnt Signaling Pathway
A_73_11	XM_5805	DKK1	5.4	7.0	1.60	ENSBTAT0000	Dickkopf 1	

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log 2FC	Ensembl transcript ID	Description	Functional categories
6484	72		1	0		0018901	homolog (Xenopus laevis)	
A_73_111955	NM_001100353	NXT2	3.51	2.98	-0.53	ENSBTAT00000027635	Bos taurus nuclear transport factor 2-like export factor 2	Protein transport
A_73_107907	NM_001046325	REEP2	8.92	9.75	0.83	ENSBTAT00000013281	Bos taurus receptor accessory protein 2	
A_73_113383	NM_001046186	CLDN15	6.76	6.37	-0.39	ENSBTAT00000000450	Bos taurus claudin 15	Ion transport
A_73_110168	XM_603860	KCNA3	2.11	2.65	0.54	ENSBTAT00000044864	Bos taurus potassium voltage-gated channel, shaker-related subfamily, member 3	
A_73_111549	NM_174792	FTL	14.19	13.48	-0.71	ENSBTAT00000009965	Bos taurus ferritin, light polypeptide	
A_73_108225	NM_001075654	COQ10B	7.25	8.17	0.93	ENSBTAT00000006115	Bos taurus coenzyme Q10 homolog B (S. cerevisiae), nuclear gene encoding mitochondrial protein	Respiratory chain
A_73_111620	AW656401	MT-ND2	6.87	7.54	0.67	ENSBTAT00000060548	Rep: NADH-ubiquinone oxidoreductase chain 2	

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log 2FC	Ensembl transcript ID	Description	Functional categories
							– Channa lucius, partial (5%)	
A_73_114745	NM_174158	PRND	5.56	7.92	2.36	ENSBTAT00000014624	Bos taurus prion protein 2 (dublet)	Ion homeostasis
A_73_114185	NM_001034757	FAM103A1	8.85	8.25	-0.60	ENSBTAT00000038779	Bos taurus family with sequence similarity 103, member A1	RNA-binding
A_73_103320	NM_001038550	SERAC1	2.72	3.31	0.59	ENSBTAT00000015295	Bos taurus serine active site containing 1	Extracellular matrix organization, lipid metabolic process
A_73_107738	NM_174603	SLC2A3	10.36	9.56	-0.80	ENSBTAT00000042889	Bos taurus solute carrier family 2 (facilitated glucose transporter), member 3	Glucose transport
A_73_103240	EE914885	ECI2	6.26	7.13	0.87	ENSBTAT00000020196	Rep: Peroxisomal D3,D2-enoyl-CoA isomerase – Bos taurus (Bovine), partial (7%)	Fatty Acid β-oxidation
A_73_114848	CB461688	SPOCK1	5.55	6.52	0.97	ENSBTAT00000004556	Rep: PREDICTED: sparc/osteonectin, cwcv and kazal-like	Signal transduction

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log 2FC	Ensembl transcript ID	Description	Functional categories
							domains proteoglycan 1 - Macaca mulatta, partial (90%)	
A_73_109258	NM_001077957	NSG1	7.80	8.33	0.53	ENSBTAT00000007502	Bos taurus neuron specific gene family member 1	Sterol biosynthetic process
A_73_113113	NM_001001163	ST8SIA4	6.36	6.81	0.45	ENSBTAT00000028590	Bos taurus ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4	Negative regulation of cell adhesion, axon guidance
A_73_106793	NM_001195050	C11H9orf16	11.48	11.02	-0.47	ENSBTAT00000018807	Bos taurus chromosome 11 open reading frame, human C9orf16	-
A_73_115169	NM_173907	DDC	8.14	7.47	-0.68	ENSBTAT00000052572	Bos taurus dopa decarboxylase (aromatic L-amino acid decarboxylase)	Catecholamine biosynthesis, cellular response to growth factor stimulus
A_73_113811	CB444810	-	4.61	4.18	-0.43	-	Unidentified transcripts	-
A_73_112621	NM_001040507	C13H20orf79	3.29	4.53	1.24	ENSBTAT00000006853	Bos taurus chromosome 13 open reading frame,	-

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log2FC	Ensembl transcript ID	Description	Functional categories
A_73_115119	CB446477	-	6.45	7.27	0.82	-	human C20orf79 Rep: Uncharacterized protein ENSP00000375250 – Homo sapiens (Human), partial (13%)	-
A_73_109430	AW483622	-	12.28	11.60	-0.68	-	Rep: HDC10915 – Drosophila melanogaster (Fruit fly), partial (15%)	-
A_73_115618	NM_001100379	SMPDL3B	3.95	4.52	0.57	ENSBTAT00000017279	Bos taurus sphingomyelin phosphodiesterase, acid-like 3B	Sphingomyelin catabolic process
A_73_111258	XM_608638	OTUD1	10.02	11.54	1.52	ENSBTAT00000065106	Bos taurus OTU deubiquitinase 1	Proteolysis
A_73_100488	XM_617206	ADAMTS9	6.04	6.69	0.65	ENSBTAT00000004113	Bos taurus ADAM metallopeptidase with thrombospondin type 1 motif, 9	Degradation of the extracellular matrix
A_73_114543	NM_001075141	ATP6V1D	13.28	12.67	-0.61	ENSBTAT00000056016	Bos taurus ATPase, H + transporting, lysosomal	ATPase activity, ion transport

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log2FC	Ensembl transcript ID	Description	Functional categories
							34 kDa, V1 subunit D	
A_73_108180	AW267045	PLEKHB2	6.12	5.76	-0.37	ENSBTAT00000001246	Bos Taurus pleckstrin homology domain containing, family B (evectins) member 2	Phospholipid binding
A_73_107233	XM_588298	CYP1A1	8.03	9.53	1.50	ENSBTAT00000054815	PREDICTED: Bos taurus cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 1	Steroid hydroxylase activity
A_73_118291	BE684793	-	13.94	13.55	-0.39	-	Unidentified transcripts on BTA14 position 647072–647770	-
A_73_119171	NM_001101883	PDK4	12.78	13.17	0.39	ENSBTAT00000038879	Bos taurus pyruvate dehydrogenase kinase, isozyme 4, nuclear gene encoding mitochondrial protein	Regulation of glucose and fatty acid metabolism/homeostasis
A_73_105647	NM_001077944	IQCF2	2.94	3.84	0.89	ENSBTAT00000045775	Bos taurus IQ motif containing F2	Protein binding
A_73_111748	XM_585051	MOSPD2	6.66	7.38	0.72	ENSBTAT00000024345	Bos taurus motile	Structural molecule

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Probe ID	Genbank accession	Gene symbol	CTRL	CST	Log 2FC	Ensembl transcript ID	Description	Functional categories
							sperm domain containing 2	activity
A_73_121262	EE895728	-	3.55	4.21	0.66	-	Unidentified transcripts on BTA1 position 6149999-6149302	-
A_73_114443	CB464317	-	3.21	2.04	-1.17	-	Unidentified transcripts on BTA22 position 32160057-32159132	-
A_73_100560	DV794529	FAM134B	14.40	13.33	-1.07	ENSBTAT00000021862	Bos taurus family with sequence similarity 134, member B	Sensory perception of pain

CTRL, control animals; CST, corticosteroid-treated animals.

Table options

Batch 5 was included to test the possible application of the classifier on meat cuts from retail since such a batch consists of muscle samples of DE treated animals from batch 4 stored as small meat cuts under vacuum at +4 °C up to 14 days to mimic the condition of commercial samples. When comparing differentially expressed genes between controls and treated animals (Significance Analysis of Microarray, FDR 10%, FC 1.5) at D0 and D14, a highly significant concordance was evidenced ($p > 0.001$, Fisher exact test; data not shown). Besides, the successful correction of batch effect by ComBat (Fig. 3) and the correct identification obtained for all samples included in batch 5 (0 classification errors) suggest that reliable measures of gene expression and classification can be obtained up to 14 days after slaughtering even when samples are stored under commercial conditions.

A further cohort, batch 3, was analyzed to evaluate the feasibility of the gene expression classifier under field conditions since batch 3 consists of samples collected at commercial slaughterhouses by unskilled staff and previously analyzed and classified with other approaches (Pegolo et al., 2012).

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When applied to this independently pre-processed and normalized batch, the classifier assigned 13 commercial samples to the “corticosteroid-treated” class. Within such positive samples are included all 4 individuals that were previously found to be positive for corticosteroid treatment at LC-MS/MS analyses, which showed the presence of Dex residues in the urines of these animals (Pegolo et al., 2012). For the same samples, also available was a classification between “negative” and “suspect” for corticosteroid treatment, which was based on thymus histology and hematic cortisol (Vascellari et al., 2008). The concordance between the two classifications (present study; Vascellari et al., 2008) was marginally significant using a Cohen's Kappa test (K = 0.39).

For most of the other animals included in this study the results of different analyses were also available (Cannizzo et al., 2011) and a global comparative classification table was reported in Supplementary Data 1.

4. Discussion

Disparate gene expression signatures have been proposed to identify animals treated with the same class of anabolic steroids using bovine muscle samples with little agreement in the constituent genes or reduced statistical significance. This can be due to different dosages and/or route of administration, specific compound effect or combination with GPs belonging to different classes (e.g. corticosteroids combined with androgens and/or estrogens), heterogeneity of computational pipelines and statistical methods employed in the analysis, and use of few subjects that cannot well represent individual variation and lead to the identification of robust and reliable biomarkers. Besides, neither gene expression signatures nor gene sets identified in individual studies have been validated so far in subsequent trials either for sample classification or for development of diagnostic screening tests.

In human medicine, when individual studies are too small to yield general conclusions, meta-analysis or integrated analysis have been adopted to encompass transcriptomic data generated with similar experimental procedures across different studies. In particular, in the field of cancer research, such approaches have been used to find diagnostic and prognostic biomarkers with sufficient sensitivity and specificity for use in a clinical setting (Phan et al., 2012), since they increase power, reduce risk of error and facilitate exploratory analysis, which generate hypotheses for future research (Gotzsche, 2000). Furthermore, the identification of biomarkers for tumor development, progression, intra- and extra-vascular invasiveness, and patient outcome led to the development of various commercial tests such as, for instance, those based on microfluidic lab-on-a-chip for genomic diagnosis (Ying and Wang, 2013) or the Breast Cancer and Colon Cancer Xpress Chip from Axela Inc (Toronto, Canada).

In the present work, for the first time in a veterinary context, the possibility was explored to apply an integrated analysis to transcriptomic data that are already available as well as to newly generated ones. The main goal was to test all the required steps toward the identification of a robust and specific signature for corticosteroid treatment in bovine muscle samples. The first step was to assess whether microarray data from different studies could be reliably analysed together, without systematic errors due to batch effects. The use of a Bayesian method for batch effect correction (ComBat) provided unbiased integrated data and normalization parameters that were used in the subsequent analyses. Such a correction was successfully implemented for samples that were collected under extremely various conditions over several years (2006–2013). The same microarray platform and labeling scheme (single-color) was used across all experiments, although microarray slide and labeling kit batches as well as lab operators varied. When microarray data using the same

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platform, but a scanner at a different resolution in a different lab (De Jager et al., 2011) were included, batch correction was much less reliable (data not shown). Therefore, different scanning protocols and inter-laboratory variation might be a limitation for robust meta-analysis. The second step was to find a high-performing classifier that could correctly classify samples either as negative or as positive based on gene expression data. A SVMs method with linear kernel showed the best performance on the dataset in terms of precision in features selection, stability of biomarkers lists and classification accuracy. Such a gene expression classifier based on SVM linear kernel consisted of 73 gene markers.

It is worth noting that the average MCC (Fig. 5) is quite stable for a number of features going from 15 to 80, with a maximum MCC in correspondence to 73 selected features. A more conservative choice would have been to select 15 features. Since, however, the objective of this work was to test all the required steps toward the identification of a robust and specific signature for corticosteroid treatment in bovine muscle samples, we preferred to consider the larger list, so as to assess in future studies the reproducibility of specific signals.

The present study was aimed at developing an operative diagnostic tool; however, it also provided statistically robust evidence about the effects of corticosteroids on bovine skeletal muscle at the transcritpome level that are common across various compounds, animal breeds and ages. Several marker genes showed a biological role linked to corticosteroid function in skeletal muscle. For instance, RAS dexamethasone-induced 1 (*RASD1*) (Table 1) encodes a member of the Ras superfamily of small GTPases critically involved in cellular control of proliferation, adhesion, differentiation and apoptosis (Tu and Wu, 1999) and it is known to be induced by Dex. *SNAI2*, which is over-expressed in corticosteroid-treated animals, is required for skeletal muscle regeneration (Zhao et al., 2002). As discussed elsewhere (Carraro et al., 2009), the effects of corticosteroids on skeletal muscle are controversial, but at low doses, they appear to promote muscle repair. Muscle regeneration involves the activation of cell cycle and proliferation. Several marker genes have been reported to be part of cell cycle progression mechanisms (Table 1). Of these, *ARRDC3* has also been shown to be required for positive control of body mass (Patwari et al., 2011). The biomarkers list comprises also many genes that participate in inflammatory/immune response (i.e. nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (*NFKBIA*), chemokine (C-C motif) ligand 2 (*CCL2*), IKAROS family zinc finger 2 (*IKZF1*), regulatory factor X, 2 (*RFX2*), TNF receptor-associated factor 5 (*TRAF5*), prostacyclin synthase, nitric oxide synthase 2 inducible (*NOS2*). Such evidence is in agreement with the known role of corticosteroids in inflammation and immunity. *NOS2* is particularly interesting as it was reported to be required for muscle regeneration (Rigamonti et al., 2013). *NOS2* regulation by corticosteroids is quite complex, but in smooth muscle cells it has been shown to be up-regulated at the mRNA level by DEX (Thakur and Baydoun, 2012). Other biological processes are represented in the genes list (Table 1), such as cell migration, regulation of transcription, Wnt signaling, respiratory chain, lipid metabolism, which are known to be relevant in the skeletal muscle cell biology.

The main finding of the present study, however, is that a relatively limited set of genes is able to discriminate between controls and corticosteroid-treated animals, despite different breeds, animal ages, and combination/doses of hormones were analyzed. The accuracy of such gene expression classifier across different sample batches was extremely good, as all samples used in the training step were correctly classified (100% accuracy), although, as stated above, its expected MCC on external, previously unseen samples, obtained by averaging the MCCs obtained on test samples across the B = 100 bootstrap runs, is equal to 0.77. It is worth noting that, with the adopted

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bootstrap schema, at each run the test samples are used only to monitor the classification error, not for the learning, and also the preprocessing steps are run independently. The average MCC across the 100 bootstrap unseen test samples as a measure of accuracy. The final classifier is then built on more examples than those used in each bootstrap run, and therefore the estimated MCC might be a sort of pessimistic estimate of the actual classification accuracy at least in case of a very defined and carefully controlled process, where the same microarray platform and labeling scheme is used across all experiments. As a proof of concept, we also chose 15 samples at random from the 89 samples and run the entire preprocessing and bootstrap procedure on the remaining 74 samples. The estimated classification accuracy obtained in this case for the final classifier in terms of MCC is 0.70. The observed MCC on the 15 samples from the validation set is 0.87, with just 1 treated sample misclassified as a control.

As already mentioned, the training set included sampled stored under conditions similar to commercial meat cuts, thereby broadening the potential for gene expression markers to the end of the production chain. It should also be noted that corticosteroid-positive samples included animals treated orally with prednisolone and all of them were correctly classified as corticosteroid-positive. Prednisolone has been reported to be difficult to identify using either direct or indirect methods (Cannizzo et al., 2011). In fact, orally-administered prednisolone yielded substantial growth-promoting effects in the animals included in the present analysis (group P of batch 1), whereas thymus in treated animals showed no histological alteration and residues of prednisolone and/or of its main metabolite, prednisone, were undetectable after few days of suspension, either in the liver or in the urine, even using highly sensitive LC-MS/MS methodologies (Cannizzo et al., 2011). Therefore, the fact that some “unknown” commercial samples, which were negative either by thymus histology or by LC-MS/MS detection of urine residues, were classified as putatively corticosteroid-treated based on gene expression, does not mean necessarily that these samples should be considered as false positives detected using the transcriptomic analysis. It might well be possible that gene expression profiling is able to identify positive samples that are false negatives using other approaches. It is actually advisable to use complementary diagnostic methods, both direct and indirect, although analysis costs might become an issue.

The proposed approach of establishing a reliable classifier through integrated analysis of a large set of transcriptomic data appears extremely promising, considering the high accuracy and robustness against potentially confounding variables (*e.g.* animal age, breed, association with additional GPs).

A possible limitation of our gene signature rise from the relatively low expression level of 15 genes out of 73 selected, which have log2 expression level lower than 5 in both treated and control samples. The main issue we see for these genes is the reproducibility issue, since variance is generally large at low levels of expression. As stated above, these genes were selected as the top 73 ranking signals (in average across the 100 bootstrap runs) among the 20,602 analyzed genes; thus they seem quite robust signals. Moreover, to be robust against variance the training and the test data in each bootstrap sample undergoes the preprocessing independently; parameters of ComBat and quantile normalization are saved and then used to normalize the test set as explained in Section 2.5.

When different protocols and/or platform are used, the reproducibility issue could be critical. Our aim here, however, was to identify a list of possible biomarkers to be used within a very defined and carefully controlled process, where the same microarray platform and scanning protocol is used across all experiments, although microarray slide and labeling kit batches as well as lab operators can vary. Inter-laboratory variation also appears to seriously affect reproducibility.

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What remains to be done to move toward routine analysis of commercial samples using such gene expression classifier as a screening tool? There are two potential frameworks where to apply indirect biomarkers to prevent GP abuse. The first one is to use them in internal controls to guarantee food quality and safety along the food supply chain. For instance, one of the largest retail chains in Italy has autonomously implemented routine histology tests to control their suppliers of beef meat and reduce the risk of GP residues in commercialized meat cuts. Under this scenario, the current level of predicted accuracy (5% FP and 6% FN) on unknown samples unrelated to those used as training set is probably sufficient already. The next step would be to focus on reproducibility, speed, and reduced costs for routine assays. The second potential application of indirect biomarkers is as a screening tool during official controls, to increase the effectiveness of direct detection of GP residues, focusing on suspect cases, and to discover GP abuse that might be undetectable with direct methods and/or traditional indirect ones as mentioned above. In the latter case, however, confirmatory analyses, which are required for suspect non-compliant cases reported with screening methods (Commission Decision 2002/657/EC), might not be effective on the non-compliant sample(s). On the other hand, closer monitoring of producers involved in such non-compliant cases might lead to confirmatory evidence. The legislation for official use of indirect markers, however, is still quite limited. The most explicit mention of indirect evidence for GP abuse is included in the recommendations of the International Federation of Horseracing Authorities (2014), which state that "the finding of any scientific indicator of administration or other exposure to a prohibited substance is also equivalent to the finding of the substance". However, to our knowledge, it is not clear how such scientific indicator(s) should be evaluated, which is the required accuracy, and how indirect methods should be validated and routinely implemented. The World Anti-Doping Agency (2013) mentioned in its Athlete Biological Passport guidelines that indirect "markers" are accepted and issued rigorous technical descriptions on standard operating procedures for sample collection and analysis, as well as for the interpretation of results. For several reasons, however, such guidelines might not be applicable to GP abuse in farming animals. Although they were conceived for direct detection methods, indications contained in the Commission Decision 2002/657/EC might be used as a first suggestion on requirements for screening methods with indirect markers. Chapter 2.2 of Technical Annex reports that "only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate of <5% (β-error) at the level of interest shall be used for screening purposes in conformity with Directive 96/23/EC". Therefore, even setting aside the need for documented validation of the method, the transcriptomic classifier proposed here has a predicted accuracy on unknown samples (new samples collected and analyzed after the classification algorithm has been set-up) that is still marginally higher (6% of false compliant cases) than the required limit (<5%).

5. Conclusions

The present work allowed to identify a panel of robust biomarkers for corticosteroid treatment in beef cattle. If gene expression markers are to be used for internal control along the supply chain, efforts should be directed toward the development of a cost- and technically-effective test. For its use as official screening method, however, additional known samples (experimentally treated and especially controls) should be analyzed to set up a more accurate classifier, which could match the requirement of <5% false compliant cases. In parallel, it would be extremely useful to better define a regulatory framework for indirect, gene expression markers, through discussion with all involved stake-holders and experts in the field.

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Conflict of interest

The authors declare no competing financial interest.

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