Transcriptomic profiling as a screening tool to detect trenbolone treatment in beef cattle

Research in Veterinary Science 96 (2014) 472–481

http://dx.doi.org/10.1016/j.rvsc.2014.03.020

Transcriptomic profiling as a screening tool to detect trenbolone treatment in beef cattle

S. Pegolo [a,*], F.T. Cannizzo [b], B. Biolatti [b], M. Castagnaro [a], L. Bargelloni [a]

[a] *Department of Comparative Biomedicine and Food Science, University of Padua, Viale dell'Università 16, 35020 Legnaro, Padova, Italy*

[b] *Department of Animal Pathology, University of Turin, via L. da Vinci 44, 10095, Grugliasco, Italy*

A R T I C L E I N F O *Article history:*

Received 19 August 2013
Accepted 28 March 2014
Keywords:
DNA-microarray
Hormone implants
Bovine
Trenbolone

A B S T R A C T

The effects of steroid hormone implants containing trenbolone alone (Finaplix-H), combined with 17 β - oestradiol (17 β -E; Revalor-H), or with 17 β -E and dexamethasone (Revalor-H plus dexamethasone *per os*) on the bovine muscle transcriptome were examined by DNA-microarray. Overall, large sets of genes were shown to be modulated by the different growth promoters (GPs) and the regulated pathways and biological processes were mostly shared among the treatment groups. Using the Prediction Analysis of Microarray program, GP-treated animals were accurately identified by a small number of predictive genes. A meta-analysis approach was also carried out for the Revalor group to potentially increase the robustness of class prediction analysis. After data pre-processing, a high level of accuracy (90%) was obtained in the classification of samples, using 105 predictive gene markers. Transcriptomics could thus help in the identification of indirect biomarkers for anabolic treatment in beef cattle to be applied for the screening of muscle samples collected after slaughtering.

1. Introduction

Food safety has become of great importance for different stakeholders in the meat industry, from farmers to consumers. In particular, the use of hormonal growth promoters (GPs) in foodproducing animals still raises concern due to the presence of residues in meat products, resulting in a potential risk for human health. The use of GPs has been banned in food-producing animals in the European Community since 1988. Among the banned substances are compounds having a hormonal or thyrostatic action and β –agonists, which have specifically been prohibited as growthpromoting agents in livestock breeding by the Council Directive 96/22/EC and subsequent modifications (Council Directives 2003/74/EC and 2008/97/EC). The Council Directive 96/23/EC established the measures to monitor the illegal use of GPs in foodproducing species and residues in both live animals and animal products and required the adoption of National Monitoring Plans by EU member States. Despite this strict regulation, hormonally active substances are still used in meat production since they increase body weight and feed conversion rates, with major economic implications. The positive effects of sexual steroids on muscle mass in mammals are exerted both directly, through intracellular receptors in skeletal muscle, and indirectly, by stimulating growth hormone release from the pituitary and other growth factors from different organs (Cantiello et al., 2008). Testosterone (T) stimulates the increased incorporation of amino acids into proteins, thus increasing muscle mass without a concomitant increase in adipose tissue (Sinha-Hikim et al., 2003). Oestrogens seem to act via stimulation of the somatotropic axis to increase growth hormone and thus IGF-1 production and availability (by modulation of the IGFbinding proteins) and were proven to stimulatemyogenic repair and regeneration (Enns and Tiidus, 2010). In addition to the use of naturally occurring steroids, synthetic compounds are often administered aswell. Trenbolone acetate (TBA) is a synthetic androgen with greater anabolic activity than T. The 17 β -TBA metabolite is also a potent androgen and reproductive toxicant (Gray et al., 2006; Hotchkiss et al., 2007), and has a higher affinity for the T receptor than endogenous T (Ankley et al., 2003). Besides, synthetic glucocorticoids (GCs) such as dexamethasone (DEX) were reported to be administered in cocktails with β -agonists and/or sexual steroids, due to their interactions at the target receptor level (Odore et al., 2007) and their consequent synergistic effects (Courtheyn et al., 2002). *In vitro* studies showed that DEX induces the proliferation of skeletal muscle cells via insulin and insulin growth-factor 1 (IGF-1; Giorgino and Smith, 1995). Analytical methods are crucial for detecting illegal residues. To

ensure reliable results, all analyses should be conducted by accredited laboratories using analytical methods validated according to the criteria and procedures established by the Commission Decision 2002/657/EC and subsequent amendments (which implemented the Council Directive 96/23/EC). Currently, the existing official targeted analyses for the identification of TBA or TBA metabolites in bovine urine samples are represented by ELISA methods for screening and Gas Chromatography (GC)- or Liquid Chromatography (LC)–Mass Spectrometry (MS)/MS methods for confirmation of the presence of residues (Italian Monitoring Plan 2013, PNR). However, the real importance of GP abuse in beef cattle appears to be underestimated because the official confirmative analytical methods are only used on a small number of cases and often appear to not be sufficiently sensitive to detect low residue levels (Nebbia et al., 2011). Moreover, official controls are eluded through the development of new hormonal compounds or the administration of GPs in cocktails which mix low doses of different molecules with similar biological effects. As the illicit administration of growth promoters is constantly evolving to evade controls, novel screening methods are necessary. The feasibility of indirect approaches, based on the assessment of perturbations in different biological systems, has been explored to identify GP-treated animals. In particular, the potential of transcriptomics to identify the effects of anabolic agents and to be used as a screening tool to inform confirmative analysis on chemical residues has been investigated in previous studies (e.g. Lancova et al., 2011; Pinel et al., 2010; Riedmaier et al., 2009). Transcriptomic profiling might provide a reliable and precise signature of treatment with GPs showing virtually any alteration in gene expression and defining an extensive portrait of the biological response to toxicants; this could help with the identification of indirect biomarkers of illicit anabolic treatment. Thus, DNA-microarray platforms have been successfully used to investigate the effects of different hormonal treatments in beef cattle (Cannizzo et al., 2013; Carraro et al., 2009; De Jager et al., 2011; Pegolo et al., 2012; Rijk et al., 2010). RNA-sequencing technology was also recently applied, in order to detect the illegal use of GPs in cattle and was proposed as a screening method to identify potential biomarkers of treatment (Riedmaier et al., 2012). Here, DNA-microarray analysis on skeletal muscle was used to evaluate the biological effects of different GP treatment protocols, using TBA alone or in combination with 17β-oestradiol (17β-E), or TBA combined with 17β-E and DEX. The potential for transcriptomic profiling as a screening tool for the detection of illicit hormonal treatment was also explored.

Material and methods

2.1. Animals and experimental design Thirty-seven Friesian cattle, all males, aged 12–25 months and weighing 450–700 kg, were randomly divided into four groups. Group F (n = 8) animals were chronically exposed for 92 days (from day 1 to day 92) to a steroid hormone implant containing 200mg TBA (Finaplix-H). Group R (n = 8) animals were chronically exposed for 89 days (from day 1 to day 89) to a steroid hormone implant containing 200mg TBA and 20 mg E (Revalor-200, Intervet, USA). Group RD (n = 8) animals were chronically exposed (89 days) to a Revalor- 200 implant (from day 1 to day 89) and were also orally administered with DEX (0.7 mg/day/animal) from day 45 to day 84. Group C (n = 13) consisted of untreated animals and served as control. The implants were placed under the skin on the back side of the ear, as per the manufacturer's recommendations (http://www.merckanimal- health-usa.com/products/130_120723/productdetails _130_121283.aspx; http://www.merck-animal-health-usa.com/ products/130_120676/productdetails_130_121148.aspx). Little information is available about the kinetics of the hormones released by the implants. Vogt and Oehrle (1977) found that, 40 days after the implantation of Implix (20 mg oestradiol plus 200mg testosterone), the hormone levels in the urine (in particular those of oestradiol) were similar to that of controls. Thus, at the time of slaughter, about 70 days after implantation, the hormone residues should have been at physiologic levels. Henricks et al. (1997) measured the serum concentration of both oestradiol and TBA in implanted heifers, and found that it was significantly higher in the first 56 days after implantation, and then declined afterwards. Similar results were obtained by the same author (Henricks et al., 1982) in a previous experiment with higher doses of hormones in the implants, which induced greater TBA and oestradiol serum levels in the first 60 days, with a subsequent considerable decrease in concentration. Likewise, heifers implanted with Finaplix-H showed a decrease in serum concentration of TBA from 300 to 400 pg/ml of the first weeks to concentrations of 90 pg/ml at d84, 64 pg/ml at d112 and 32 pg/ml at d140 (Henricks et al., 1997). Also, after 8 weeks of exposure to Finaplix-H treatment, the maximum residue limit (MRL) of 10 ng 17α-TBA/g liverwas exceeded with a 10-fold dose in animals that were experimentally treated and a 3-fold dose in one animal. In muscle, no violation of the MRL (2 ng/g) was observed with the highest detectable concentration of 0.3 ng/g after 10-fold treatment. Maximum values of 1.4 ng 17α-TBA/g were determined in kidney and 2.5 ng 17β-TBA/g in fat at the highest dosage (Lange et al., 2001). No recommendations regarding food safety have been made by the manufacturers, either on the product label for Revalor-200 (http://intervetus.naccvp.com/product/view/1047415) or for Finaplix-H (http://intervetus.naccvp.com/product/view/1047325) implants. All

groups of experimental animals were kept in separate boxes (10m × 15 m), andwere fed with a diet consisting of corn silage, corn, hay and a commercial protein supplement; water was supplied ad libitum. The animals were housed in ventilated stables, and the experiment was carried out in accordance with the EU animal welfare legislation. Animalswere slaughtered 5 days after the last DEX treatment, and the implant remained in place until euthanasia. The experiment was authorised by the Italian Ministry of Health and the Ethics Committee of the University of Turin. Carcasses of treated animals were appropriately destroyed (2003/74/CE – DL 16 March 2006, n. 158).

2.2. Animal growth performance and health status The beef cattle were weighed monthly at four times: T0 (day 0), T1 (day 36), T2 (day 61) and T3 (day before slaughtering). The average daily gain (ADG) was calculated as the difference between two subsequent body weights. Health status was monitored daily by recording all individual pathological events and medical treatments.

2.3. RNA extraction Total RNA was extracted from *biceps brachii* skeletal muscle samples stored in RNA later (Life Technologies, NY, USA) at −20 °C using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, samples (30 mg) were disrupted and homogenised using buffer RLT and β-mercaptoethanol in the Ribolyzer sample homogeniser (Bio- Rad, Milano, Italy). Proteinase K (Qiagen, Hilden, Germany)was added and after incubation for 10 min at 52 °C, samples were centrifuged for 3 min at full speed. The supernatant was transferred into a new tube and 70% ethanol was added; the samples were then transferred into the RNeasy spin columns and centrifuged for 30 s at 10,000 rpm. After discarding the flow-through, RW1wash bufferwas added, sampleswere centrifuged for 30 s at 10,000 rpm and the flowthroughwas discarded. DNase I (Qiagen, Hilden, Germany)was added and samples were incubated for 15 min at RT to remove any DNA contamination. RW1 buffer was added, and the tubes were centrifuged for 30 s at 10,000 rpm. Then, RPE buffer was added and the tubes were centrifuged for 30 s at 10,000 rpm. A second wash with RPE buffer was performed and samples were centrifuged for 2 min at 10,000 rpm, discarding the flow-through. To remove any ethanol residue, the tubes were centrifuged again for 1 min at full speed. Elution of total RNA was performed by placing the column in a new tube and adding 30 _l of DNase/RNase free water; the column was incubated for 1 min, and then centrifuged for 1 min at 10,000 rpm. To maximise yield, the flow-through was reloaded into the column and eluted again by centrifuging the tubes for 1 min at 10,000 rpm. The concentration of RNA samples was measured using a UVvis spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE), and RNA integrity was estimated by running each sample on an RNA-chip in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA integrity number (RIN) was evaluated using the Agilent 2100 Expert software, and this was used as a measure to standardise the interpretation of RNA quality, which is no longer determined by the ratio of the ribosomal bands, but by the entire electrophoretic trace of the RNA sample. In the present study, a conservative threshold was enforced in order to reduce experimental biases due to poor RNA quality. Only RNA samples with an RIN number ≥7.0 were included in the analysis.

2.4. RNA amplification, labelling and hybridisation Sample labelling and hybridisation were performed according to the Agilent One-Colour Microarray-Based Gene Expression Analysis protocol. Briefly, for each sample, 200 ng of total RNA was linearly amplified and labelled 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 labelling in order to monitor microarray analysis workflow. Labelled cRNA was purified with the Qiagen RNAeasyMini 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 labelled cRNA was prepared for fragmentation by adding 11 μL 10 × Blocking Agent and 2.2 μL of 25 × Fragmentation Buffer; this was heated at 60 °C for 30 min, and finally diluted by the addition of 55 μL 2 × GE Hybridization buffer. A volume of 100 μL of hybridisation solution was then dispensed onto the gasket slide and this was assembled with the microarray slide (each slide contained 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 synthesised 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 hybridisation chamber, quickly submerged in GE Wash Buffer 1 to disassemble the slides and then washed in GEWash Buffer 1 for approximately 1 min; this was followed by one additional wash in pre-warmed (37 °C) GEWash Buffer 2. Hybridised slides were scanned at a resolution of 5 μmusing an Agilent G2565BA DNA microarray scanner. Default settings were modified to scan the same slide twice using 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 the GEO Series accession number GSE47827.

2.5. Normalisation of microarray data The two linked images generated from the scanning processwere analysed together; data were extracted and the background signal was subtracted using the standard procedures found in the Agilent Feature Extraction (FE) Software version 9.5.1. The Feature Extraction Software returns a series of spot quality measures to evaluate the goodness and the

reliability of spot intensity estimates. All control features except spike-in (Spike-In Viral RNAs) were excluded from subsequent analyses. To avoid possible bias due to sample processing and slide variation, Spike-In Viral RNAs were added to each sample at the beginning of the microarray experiment to provide an internal quality control. Each spike-in RNA has a different known concentration following a dilution series and there are 32 replicate probes for each spike-in RNA on the array. Spike-in control intensities were used to identify the best normalisation procedure for each data set. After normalisation, spike intensities are expected to be uniform across the experiments of a given data set. Based on the uniformity of the spike-in intensities across the samples, the cyclic Loess normalisation procedure was chosen. After normalisation, a further quality control step was performed by removing all probes with intensity values lower than the second lowest spike-in concentration (3.5) in at least 80% of the samples as this value was considered too close to the limit of detection. Filtering and normalisation procedures were performed using R statistical software, which is available at http://www.r-project.org/.

2.6. Statistical analyses To explore the effect of age, weight at T0 and treatment on ADG calculated between T0 and T3 (the day before slaughtering), the oneway analysis of variance (ANOVA) test was performed with the level of significance set at $p < 0.05$, using SPSS Statistics 20 software (IBM, Armonk, New York, USA). A two-way analysis ANOVA test was performed using the TMEV suite (Saeed et al., 2003, 2006), considering treatment (F, R and RD) and age (<16 months, ≥16 months) as categorical independent variables, in order to identify lists of genes significant for age, treatment or the interaction age*treatment (level of significance set at $p < 0.05$). Sample class prediction was carried out using the program Prediction Analysis of Microarray (PAM; Tibshirani et al., 2002), which is available online at http://www.stat.stanford.edu/~tibs/PAM. *2.7. Functional enrichment analysis* Enrichment analysis on differentially up- and down-regulated genes was performed using the Functional Annotation tool that is available in the DAVID Database (http://david.abcc.ncifcrf.gov/). All GO terms and KEGG pathways included in the DAVID knowledgebase were considered. For KEGG terms, the following parameters were used: gene count 4, ease 0.05. For GO, Biological Process and Molecular Function (BP_FAT and MF_FAT, respectively), were used with gene count 4 and ease 0.05. *2.8. Meta-analysis* Gene expression data for the R group experiment were analysed together with those from another study (De Jager et al., 2011), where the effects of the same implant (trenbolone acetate plus 17β-E) in the *longissimus dorsi* muscle from 48 Brahmans cattle were analysed using the same DNA microarray platform as in the present paper. Raw data were downloaded fromthe GEO database (GSE25005) and normalised by the cyclic Loess normalisation procedure, together with raw data from the present study. Probes with fluorescence values lower than the second lowest spike in concentration (<10) in at least 50 samples were filtered out. Finally, a further tuning step was then performed by conducting a two-way ANOVA test, using treatment and site (the experiment was conducted at two sites, with different diets and treatment durations) as independent variables, on expression data fromDe Jager et al. (2011). PAM was then applied to expression data from the treatmentsignificant genes in common between the two experiments for a total of 69 samples, in order to assess the ability to classify the samples.

Table 1
Overall weight gain and ADG calculated between T0 and T3.

	LIVEWEIGHT (kg)				ADG (kg/day)			
	T0	T1	T2	T3	T0-T1	T1-T2	T2-T3	T0-T3
GROUP R	462.6 ± 77.4	524.1 ± 67.5	569.6 ± 71.3	612.1 ± 70.9*	2.05	1.52	1.42	1.66 ± 0.16[a]
GROUP RD	338.6 ± 30.9	385.8 ± 28.1	437.3 ± 34.8	462.5 ± 40.6	1.57	1.72	0.84	1.42 ± 0.16
GROUP F	480.6 ± 87.4	480.6 ± 87.4	583.1 ± 96.8	611.0 ± 94.3	0	3.42	0.93	1.45 ± 0.23
GROUP C	471.7 ± 57.4	521.2 ± 53.5	559.7 ± 55.4	594.1 ± 52.4	1.65	1.28	1.15	1.36 ± 0.32

R: Revalor; RD: Revalor +Dexamethasone; F: Finaplix; ADG: Average Daily Gain.
* $p < 0.05$, T3-T0 significantly different from the control.
[a] $p = 0.053$, significantly different from the control.

3. Results

3.1. Animal health status and growth performance The health status of all experimental animals was satisfactory during the entire experimental period and no specificmedical treatment was necessary for any individual. In comparison with the control group, overall weight gains measured just before slaughtering were larger in animals of group R (22.2%, $p < 0.05$), group F (6.5%) and group RD (4.1%), indicating that cattle responded to exposurewith exogenous hormonal growth promoters and confirming the potential anabolic effects of trenbolone, 17β-E and DEX (Table 1). ADG calculated between T0 and the day before slaughtering (T0-T3) was higher in all treatment groups with respect to controls, although the difference was only marginally significant for R-treated animals ($p = 0.053$; Table 1). Age and weight at T0 did not significantly affect ADG.

3.2. Microarray data quality control High quality, unbiased and reproducible gene expression data are essential in any DNA microarray experiment. To this end, the use of spike-in control RNAs, which have a known concentration and are processed as target RNAs, allows the robustness of the entire analysis to be controlled, including labelling, hybridisation and scanning. Comparison of normalised, averaged spike-in signals across different experimental replicates provided a strong indication that variation across samples was minimal. All samples (negative and treated samples) were also normalised together in a single run to avoid potential biases. A further filtering processwas performed with the second lowest spike-in concentration set as the limit of detection, in order to prevent the inclusion of low signal probes. This resulted in the removal of 4141 unique transcripts.

3.3. Microarray data analyses After data extraction, normalisation and filtering, processed signals for 17,333 unique transcripts in 37 muscle samples were analysed. In the present study, a wide age range was used in the experimental groups to represent, at least in part, the biological variation that naturally occurs. The application of a two-way ANOVA test, which considered age and treatment as independent variables, allowed lists of genes that were significant for both factors to be obtained as well as their interaction for each treatment group (Table 2). A comparison among treatment-significant genes for all of the experimental groups is reported in Fig. 1. Enrichment analysis using the Functional Annotation tool in DAVID identified a large set of GO terms and KEGG pathways that are significantly enriched in the F group. Of note are several genes involved in the cell cycle (21 genes) and cell division (11). Significant enrichmentwas also observed for the “cytoskeleton organisation process” (13) and for the “regulation of transcription” (51). In addition, several ribosomal proteins (10) were differentially expressed (ribosomal protein L29, L35, L36, S3, S5, S14, S16, S27a, S28, ubiquitin C; see Supplementary data 1 in the online version at doi:10.1016/j.rvsc.2014.03.020). For the R treatment group, functional annotation analyses revealed more than 90 GO terms and 12 KEGG pathways to be significantly enriched (see Supplementary data 2 in the online version at doi:10.1016/j.rvsc.2014.03.020). Finally, functional annotation for the RD treatment group evidenced more than 50 GO terms that were significantly enriched and the regulation of KEGG pathways related to the spliceosome (24 genes), glycolysis/ gluconeogenesis (13 genes) and the MAPK (36 genes) and Notch (9 genes) signalling pathways (see Supplementary data 3 in the online version at doi:10.1016/j.rvsc.2014.03.020). Expression data of ANOVA treatment-significant genes from negative controls and experimentally treated animalswere also analysed using a statistical approach for class prediction implemented in the PAMsoftware to evaluate the ability to classify samples. A discriminant analysis was first performed on selected samples (controls and treated animals; Training Sample Set) to choose the smallest panel of genes that provides the greatest accuracy of class prediction (the smallest misclassification error). The accuracy of class prediction on

Table 2
Significant gene lists resulting from the two-way ANOVA test using treatment and age as categorical independent variables ($p < 0.05$).

	Treatment	Age	Treatment*Age
Group F	958	1045	1558
Group R	2695	1733	1562
Group RD	2482	1555	1088

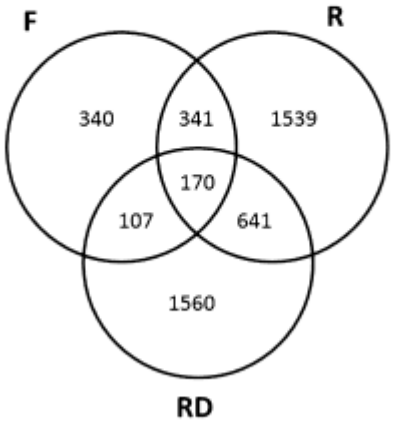

Fig. 1. Venn diagram comparison of the genes significant for the treatment variable (two-way ANOVA). F: Finaplix; R: Revalor; RD: Revalor plus DEX.

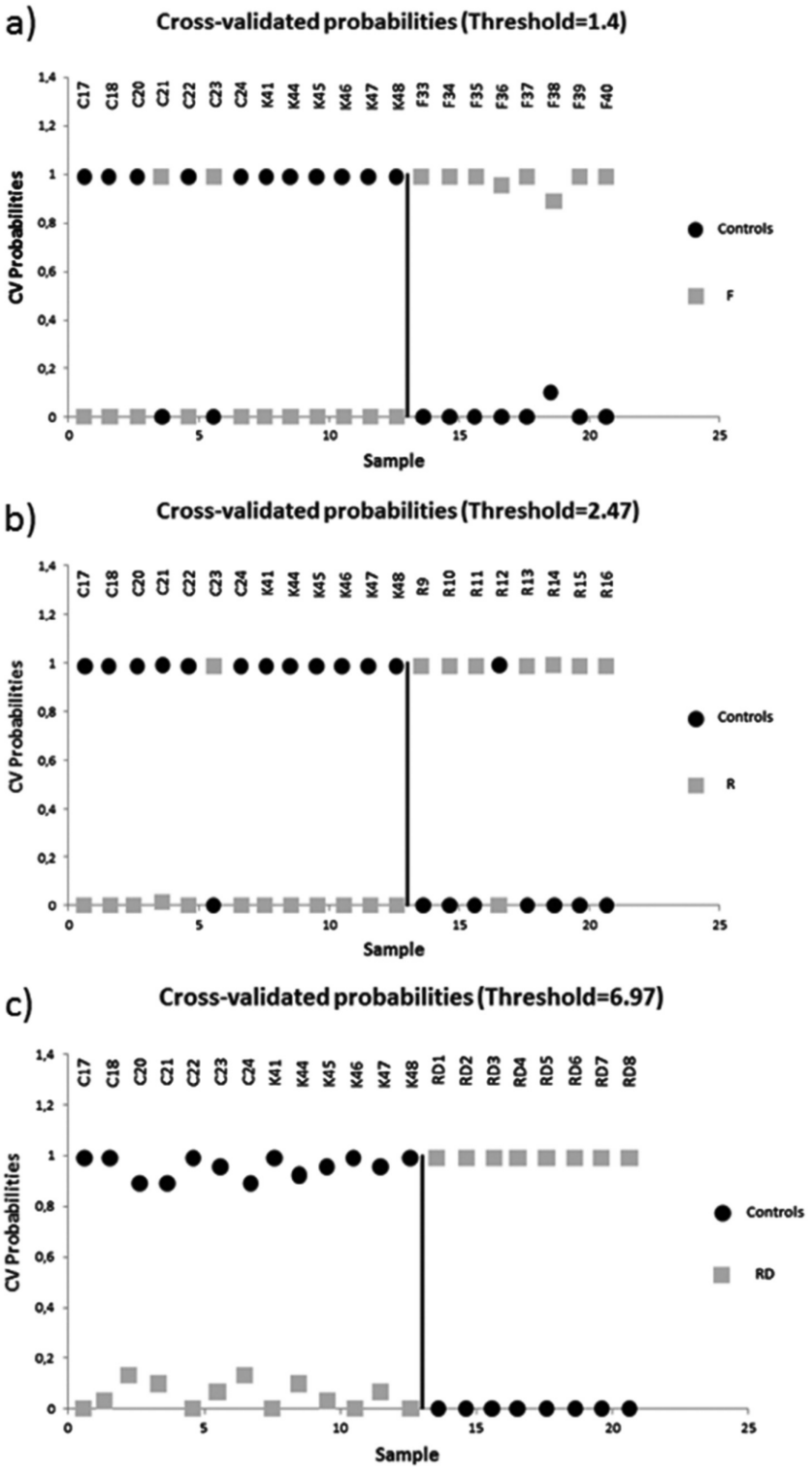

Fig. 2. Plot of cross-validated probabilities for sample classification using genes significant for the treatment variable (two-way ANOVA). (a) On the *x*-axis are individual samples: 1–13 – negative controls, and 14–21 – Finaplix-treated animals (F); on the *y*-axis, the probability of being classified as controls (circles) or treated (squares) is indicated;
training error 0.05. (b) On the *x*-axis are individual samples: 1–13 – negative controls, and 14–21 – Revalor-treated animals (R); on the *y*-axis, the probability of being classified as controls (circles) or treated (squares) is indicated; training error 0.05. (c) On the *x*-axis are individual samples: 1–13 – negative controls, and 14–21 –Revalor plus DEX-treated animals (RD); on the *y*-axis, the probability of being classified as controls (circles) or treated (squares) is indicated; training error 0.

the Training Sample Setwas then estimated through cross-validation (10% of samples were randomly extracted and classified based on the discriminant function calculated on the remaining cases). For the F treatment group, PAM enabled the two classes (controls and treated animals) to be exactly discriminated, except for two control animals, using 279 genes out of a total of 958 (Fig. 2a). With

expression data from the R treatment group, an exact classification was achieved with 331 genes out of 2695 for all samples, except for one control and one treated animal (Fig. 2b). Finally, controls and RD-treated animals were exactly classified with a probability close to 1, using only 8 genes out of 2482 (Fig. 2c). *3.4. Meta-analysis* To evaluate the possibility of extending sample classification to additional external samples, data from a previous study (De Jager et al., 2011) on the effects of an R implant in a different breed and muscle type were retrieved and analysed together with controls and animals from group R which were examined in the present paper. After normalisation and quality filtering, expression values were obtained for 18,555 transcripts in 69 samples. The application of a two-way ANOVA test, as described above, on the expression data from De Jager et al. (2011), yielded a list of 2056 genes that were significant for the "treatment" variable, while the same test on the R group in the present study identified 2695 treatment-significant genes. Comparing these two lists, 306 genes were common to both. PAM analysis was repeated using expression data for these 306 genes and cross-validation showed quite accurate classification, as 90% of sampleswere correctly classified using 105 genes (Fig. 3; see Supplementary data 4 in the online version at doi:10.1016/j.rvsc.2014.03.020).

4. Discussion The present study aimed to evaluate the effects of three different treatment protocols, all including an androgenic compound, on gene expression in the bovine skeletal muscle, in order to identify potential biomarkers for illicit treatment. Identification of transcriptomic signatures of biological conditions is an important issue in different fields such as cancer genomics, toxicogenomics or environmental genomics. Transcriptomic data have the ability to provide an integrative representation of the various biological processes and signalling pathways that are affected by specific conditions in different tissues or cell types. However, biological variation between individuals as well as uncontrolled variables might alter gene expression profiles, with confounding effects on transcriptomic signatures for target conditions. In skeletal muscle, many intrinsic or extrinsic factors (e.g. age, gender, muscle type, castration, breed, diet) are known to affect gene expression (Brandstetter et al., 2000; Middelbos et al., 2009; Moreno-Sánchez et al., 2012). In the context of GP abuse prevention, it is crucial to identify those genes that are predominantly regulated by the anabolic treatment. In the present study, several of these variables were identical across samples by experimental design, whereas two main age groups were present. The influence of age on gene expression profiles was thus explored using a two-way ANOVA test, where treatment and age were treated as independent categorical variables. A considerable effect of age on gene expression data was evident, but a large list of genes that are significantly regulated by GPs treatment was also observed. Such evidence highlights the importance of extensive sampling and the careful analysis of transcriptome data, especially when translating research results into diagnostic routines. As mentioned above, transcriptomic data convey a wealth of information on cellular processes and pathways, shedding light on the biological effects of anabolic compounds.

4.1. Finaplix treatment Functional enrichment analyses on the lists of genes that were significantly regulated after Finaplix treatment suggest a shift towards cell differentiation rather than proliferation. This is in agreement with evidence that male sexual steroids can regulate the balance between cell survival, proliferation and apoptosis through the activation of transcription factors (TFs), genes involved in mitosis and cell cycle processes, or specific signalling pathways (Vasconsuelo et al., 2011). Several genes involved in cell cycle/cell division (e.g. cyclin-dependent kinase 1, cell division cycle 40, RAD21, RAD51)were affected by androgen administration, confirming previous evidence from other tissues (Heisler et al., 1997; Khandelwal et al., 2012; Pradeep et al., 2002). Significant transcriptional regulation of TFs generally has a large effect since TFs regulate the expression of a broad network of downstream genes. Among the differentially expressed TFs, several of which are similarly affected in the three treatment groups,myocyte enhancer factor-2B (MEF2B) was found to be over-expressed in androgen-treated animals. MEF2B plays a critical role in skeletal muscle differentiation and acts as an end point for diverse intracellular signalling pathways that controlmyogenesis and muscle hypertrophy (McKinsey et al., 2000). Down-regulation of the androgen receptor (AR) was also observed. Androgens are reported to decrease AR mRNA levels in both prostate cancer LNCaP and breast cancer MDA453 cells (Yeap et al., 1999), while, in contrast, in cultured satellite cells from human skeletal muscle, incubation with androgens induced very modest changes in AR mRNA levels (Sinha-Hikim et al., 2004). On the other hand, oestrogen-related receptor alpha (ERR-alpha)was up-regulated. In C2C12myocytes, ERRalpha over-expression was shown to accelerate differentiation (Murray and Huss, 2011).

4.2. Revalor treatment The combination of androgen and oestrogen (Revalor treatment) seemed to confirm a bias towards cell differentiation. The modulation of genes involved in the cell cycle (23, many of which were commonly regulated when compared with the F group), positive regulation of cell differentiation (18 genes) and negative regulation of cell proliferation (23 genes) were observed in R group animals. Among the TFs, Jun proto–oncogene (c-Jun), Jun oncogene B (JunB) and Jun oncogene D (JunD)were up-regulated in these animals. Jun proteins, through heterodimer formation with Fos, are involved in the formation of activating protein-1 (AP-1), which has been shown to be involved in the positive control of muscle differentiation (Andreucci et al., 2002; Moore-Carrasco et al., 2006). Further evidence in support of an effect of sexual steroids on the balance between cell proliferation and differentiation comes from the

involvement of the mitogen-activated protein kinase (MAPK) pathway (43 genes), the Jak-STAT signalling pathway (25 genes) and the TGF-β pathway (16 genes). MAPKs are major components of pathways controlling embryogenesis, cell differentiation, cell proliferation and cell death (Pearson et al., 2001). Jak-STAT is a key pathway controllingmyoblast proliferation and differentiation (Sun et al., 2007). Finally, cross-talk of AR signalling with the TGF-β pathway through T-cell factor 4 and follistatin was suggested to potentially explain androgen effects onmyogenic differentiation (Singh et al., 2009). Among the biological processes assessed, lipid metabolism was significantly enriched in R treatment group. This observation is in agreement with the established role of sexual steroids in the regulation of lipid metabolism in skeletal muscle. Oestradiol is known to stimulate lipolysis and consequently to increase the utilisation of free fatty acids as an energetic substrate (Svensson et al., 2010). In addition, it has been reported that a peak in circulating oestradiol in men increases β-oxidation enzymes (Maher et al., 2010).

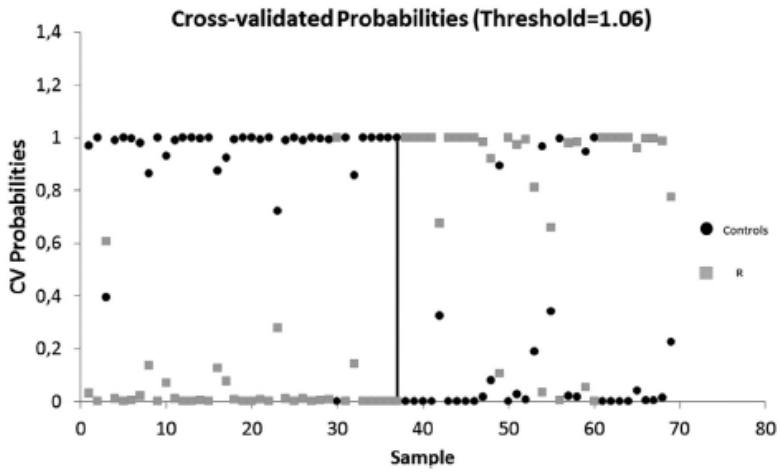

Fig. 3. Plot of cross-validated probabilities for sample classification using genes significant for the treatment variable in common (two-way ANOVA). (a) On the *x*-axis are individual samples: 1–24 – negative controls from the experiment by De Jager et al. (2011), 25–37 – negative controls from the present study, 38–61 – Revalor-treated animals from De Jager et al. (2011), and 62–69 – Revalor-treated animals from the present experiment (R); on the *y*-axis, the probability of being classified as controls (circles) or treated (squares) is indicated; training error 0.08.

T can also regulate lipolysis by influencing catecholamine signal transduction in adipocytes (Arner, 2005). In particular, it has been suggested that T inhibits lipid uptake in adipocytes and stimulates lipolysis (De Pergola, 2000). Revalor exposure was shown to affect fatty acid metabolism with 21 differentially expressed genes. In particular, peroxisomal proliferator activated receptor α (PPAR-α) was over-expressed in R group animals. PPAR-α stimulates fatty acid oxidation (Ruby et al., 2010). The hypothesis of β-oxidation activation upon Revalor administration was further supported by the observed up-regulation of the acyl-CoA oxidase 1, palmitoyl (ACOX1) gene. ACOX1 is localised to peroxisomes and is the first enzyme of the fatty acid β-oxidation pathway, which catalyses the desaturation of acyl-coenzyme A to 2-trans-enoyl-coenzyme. Likewise, upregulation of NR4A1 (or Nur77) was observed. NR4A1was reported to have a role in the regulation of lipolysis (Maxwell et al., 2005) and glucose metabolism (Chao et al., 2007) in skeletal muscle. Comparing the present results to those of De Jager et al. (2011), some common points can be underlined. For instance, up-regulation of hormone growth promoting-related genes, such as oestrogenrelated receptor alpha (ERR-alpha, both studies), or pro-myogenic transcription factors/regulators of muscle mass (myogenic differentiation 1, De Jager et al., 2011; myogenin, follistatin-like 3, myogenic factor 6, present study) was reported. In addition, the upregulation of oxytocin (OXT) after Revalor exposure was confirmed, although to a lesser extent (2.3-fold), further supporting the putative role of OXT in mediating the anabolic effect of Revalor. On the other hand, some differences emerged between the two studies. While Revalor treatment in the present experiment appeared to stimulate β-oxidation, as reported above, down-regulation of some lipid metabolism markers (not observed in our study) led De Jager and colleagues to suggest a lipid storage activity of intramuscular adipocytes. Besides, in the present study, a clear shift towards a slowfibre phenotype, as reported by Jager et al., was not observed, although the down-regulation of some genes encoding fast fibre-type subunits (myozenin 1, tropomyosin 1) was confirmed. Finally, instead of up-regulation of insulin-like growth factor 1, a limited under-expression (1.4 FC) was found in the R-treated animals in the present experiment. However, it is worth mentioning that the animals used in these experiments were different for both intrinsic (e.g. breed, age, castration) and extrinsic (e.g. diet) factors and also for the skeletal muscle sampled (*biceps brachii versus longissimus dorsii*). In addition, De Jager and colleagues evaluated the effects of the same implant, but with two treatment lengths (48–49 days and 87–88 days), and in animals reared at two different sites, and then computed the difference between the normalised mean expression of a gene in the two conditions as the measure of differential expression. What emerged

fromthese results is that considering only a small number of factors/variables with a fewanimals often makes the identification of clear differences and robust biomarkers difficult. Ametaanalysis, i.e. the inclusion of all available data from other studies or experiments, might remedy, at least in part, this drawback. Metaanalysis of transcriptomic data has already been proposed in cancer genomics, among others, providing a much broader set of data from which to make more general inferences on cancer classification (Tang et al., 2013). In the present study, gene expression data from the R experimental group and from the controlled experiment of De Jager et al. (2011)were re-analysed together, including more variables (e.g. breed, age, castration, muscle type) and a higher number of samples (69), thus potentially increasing the robustness of class prediction analysis with respect to potentially confounding variables. In fact, after data pre-processing, a high level of accuracy (90%) was obtained in the classification of samples with a set of predictive gene markers.

4.3. Revalor plus DEX Less evident was the involvement of cell cycle regulation in response to RD treatment. However, under-expression of Msh homeobox 2 (MSX2) was observed after Revalor plus DEX exposure. MSX2 is a member of a family of proteins that serve as transcriptional repressors and negative regulators of differentiation by preventing cell cycle exit and blocking the terminal differentiation of mesenchymal progenitor cells (Hu et al., 2001). In addition, up-regulation of the MAPK pathway (36 genes) was observed in the RD group. Beside the functional categories already mentioned, the combination of Revalor and DEX (RD group) showed a significant effect on specific routes in glucose metabolism, i.e. gluconeogenesis and glycogen deposition. Glucocorticoids are known to have important effects on such processes (Andrews and Walker, 1999). In addition, T was also suggested to be an important regulator of insulin sensitivity in muscle, mainly by influencing the regulation of glycogen synthesis (Holmäng, 1993). Of particular relevance is the down-regulation of genes encoding enzymes involved in the glycolysis process, which was observed in the present study (aldolase A fructose-bisphosphate, enolase 2, enolase 3, phosphoglycerate mutase 2, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase 1). Concordant evidence comes from proteomic analysis of bovine skeletal muscle (Stella et al., 2011) in response to corticosteroid abuse, since all of these enzymes were reported to be down-regulated at the protein level. These results were further confirmed by Pegolo et al. (2012), who reported the down-regulation of genes encoding three glycolytic enzymes in animals treated with corticosteroids. Also of note is the observation that two members of the olfactory receptor (OR) family, olfactory receptor family 1 subfamily E member 2-like (OR2E2) and olfactory receptor family 2 subfamily A member 1-like (OR2A1), were over-expressed in RD group animals. These results confirm that ORs might have a relevant role in myogenesis and muscle regeneration, as previously reported (Griffin et al., 2009), and supported the results obtained by Pegolo et al. (2012) in skeletal muscle from beef cattle treated with corticosteroids. In particular, OR1E2 and OR2A1 were up-regulated in that study as well, by 10- and 6-fold, respectively. The application of DNA-microarray technology allowed large lists of genes expressed in bovine skeletal muscle that are modulated by different treatment protocols with a combination of sexual steroids and corticosteroid to be obtained. Despite the fact that regulated pathways and biological processes were mostly shared among different groups, there seemed to be only a moderate overlap in the gene expression profiles, with a rather small number of genes that were commonly regulated (170; Fig. 1, Supplementary data 5 in the online version at doi:10.1016/j.rvsc.2014.03.020) when androgen is administered alone (F) or in combination with oestrogen (R) or oestrogen plus DEX (RD). Analysis of gene function did not reveal one or more specific pathways since the commonly regulated transcripts encoded proteins with various roles in cell biology (e.g. transcription factors, kinases, metabolic enzymes, phosphatases). For instance, modulation of transcripts involved in cell differentiation, such as ERR-alpha, or in glucose homeostasis regulation, such as the insulin receptor, was observed in all treatment groups. The over-expression of neurotensin 1 might be explained by the effect of sexual steroid hormones, which were shown to induce neurotensin expression in leiomyoma smooth muscle cells (Rodríguez et al., 2010). Strong down-regulation of the metabotropic glutamate receptor 8 (GRM8, around 5-fold in the F and R group and more than 10-fold in the RD group), in the skeletal muscle instead, is not easily explained. In the nervous system, glutamate receptors represent essential components in the neural transduction of positive hormonal feedback on gonadotropin secretion. Moreover, sex steroid hormones seemed to regulate the expression or responsiveness of glutamate receptors in key neural pathways ormay alter glutamate release, thereby influencing the expression and/or secretion of GnRH (Gu et al., 1999). Whether GRM8 has an additional role in muscular cells or in the muscle-innervating fibres remains to be elucidated. Overall, the limited overlap observed across genes that are significantly regulated in the three hormonal treatments could be explained by the opposing actions that androgens and oestrogens have on various endocrine targets, through a direct interaction between AR and ER (Panet-Raymond et al., 2000). Although androgens act predominantly directly through the AR, some effects are also exerted *via* the ER through the aromatisation of T to E (Mooradian et al., 1987). Axell et al. (2006) proposed that a putative balance between androgen and oestrogen levels may enhance muscle mass and strength while preserving resistance to fatigue. GCs and oestrogens have been also reported to act in opposition in the regulation of several biological processes (Zhou et al., 1989), along with GCs and androgens, due the direct physical interaction between their receptors with the mutual inhibition of AR and GR through heterodimer formation. The present study, however, was not designed to elucidate the effects of single compounds on bovine skeletal muscle, but rather to identify which genes and biological processes are affected by those GPs, either alone or in combination, which are most frequently reported in illicit practices.

5. Conclusions The arms race in the war between GP abuse and prevention requires novel tools to complement direct methods for the detection of GP residues. Transcriptomics was confirmed to be effective in providing indirect evidence of GP treatment, by revealing the biological effects of GP administration and identifying potential biomarkers when working on samples collected after slaughtering, animal carcasses or even single meat cuts. Otherwise, alterations in target organ morphology are not always evidenced by histological analyses (Cannizzo et al., 2013). The *in vivo* analysis of urine samples, looking at either directly TBA or its metabolites indirectly, is certainly more efficient for the prevention of the illegal use of GPs in farms. However, the low dosages and the rapid metabolism and excretion of these substances oftenmakes the determination of urinary residues extremely difficult, even when using targeted LC–MS methods. On the other hand, the effects of anabolic steroids on gene expression appear to persist for several days after the time of withdrawal and thus might reveal the illicit treatment even when the parental compound or its metabolites are no longer present or detectable. Nevertheless, to move from the experimental setting to the development of diagnostic methods that can be implemented on commercial samples, it is necessary to further explore the effects of biological variation and confounding factors on gene expression markers. As transcriptomic data accumulate, it becomes more and more feasible to perform broader analyses, which should provide robust gene expression signatures for the identification of suspect samples. It is clear that, at least at the present time, transcriptomics cannot replace the official targeted residual analyses, but can certainly complement the existing methods to improve the effectiveness of control strategies for the prevention and repression of the illegal use of GPs. **Acknowledgements** Thisworkwas financially supported in part by the Ministero delle Politiche Agricole e Forestali under the project 'SAFORISK', which was granted to M. Castagnaro and L. Bargelloni (D.M. 2089/09, 29 January 2009). **Appendix: Supplementary material** Supplementary data to this article can be found online at doi:10.1016/j.rvsc.2014.03.020

References

Andreucci, J.J., Grant, D., Cox, D.M., Tomc, L.K., Prywes, R., Goldhamer, D.J., et al., 2002. Composition and function of AP-1 transcription complexes during muscle cell differentiation. The Journal of Biological Chemistry 277, 16426–16432.

Andrews, R.C., Walker, B.R., 1999. Glucocorticoids and insulin resistance: old hormones, new targets. Clinical Science 96, 513–523.

Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., 2003. Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environmental Toxicology
and Chemistry 22, 1350–1360.

Arner, P., 2005. Effects of testosterone on fat cell lipolysis. Species differences and possible role in polycystic ovarian syndrome. Biochimie 87, 39–43.

Axell, A.M., MacLean, H.E., Plant, D.R., Harcourt, L.J., Davis, J.A., Jimenez, M., et al., 2006. Continuous testosterone administration prevents skeletal muscle atrophy and enhances resistance to fatigue in orchidectomised male mice. American Journal of Physiology. Endocrinology and Metabolism 291, E506–E516.

Brandstetter, A.M., Pfaffl, M.W., Hocquette, J.F., Gerrard, D.E., Picard, B., Geay, Y., et al., 2000. Effects of muscle type, castration, age, and compensatory growth rate on androgen receptor mRNA expression in bovine skeletal muscle. Journal of Animal Science 8, 629–637.

Cannizzo, F.T., Pegolo, S., Starvaggi Cucuzza, L., Bargelloni, L., Divari, S., Franch, R., et al., 2013. Gene expression profiling of thymus in beef cattle treated with prednisolone. Research in Veterinary Science doi:pii: S0034-5288(13)00125-2, 10.1016/j.rvsc.2013.03.025.

Cantiello, M., Carletti, M., Dacasto, M., Martin, P.G., Pineau, T., Capolongo, F., et al., 2008. Cytochrome P450 inhibition profile in liver of veal calves administered a combination of 17beta-oestradiol,

clenbuterol, and dexamethasone for growthpromoting purposes. Food and Chemical Toxicology 46, 2849–2855.

Carraro, L., Ferraresso, S., Cardazzo, B., Romualdi, C., Montesissa, C., Gottardo, F., et al., 2009. Expression profiling of skeletalmuscle in young bulls treated with steroidal growth promoters. Physiological Genomics 38, 138–148.

Chao, L.C., Zhang, Z., Pei, L., Saito, T., Tontonoz, P., Pilch, P.F., 2007. Nur77 co-ordinately regulates expression of genes linked to glucose metabolism in skeletal muscle. Molecular Endocrinology 21, 2152–2163.

Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, 12 August 2002.

Directive 2003/74/EC of the European Parliament and of the Council amending Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists, 22 September 2003.

Directive 2008/97/EC of the European Parliament and of the Council amending Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists, 19 November 2008.

Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of β-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/ EEC.

Council Directive 96/23/EC of 23 May 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC.

Courtheyn, D., Le Bizec, B., Brambilla, G., De Brabander, H.F., 2002. Recent developments in the use and abuse of growth promoters. Analytica Chimica Acta 473, 71–82.

De Jager, N., Hudson, N.J., Reverter, A.,Wang, Y.H., Nagaraj, S.H., Café, L.M., et al., 2011. Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fiftyfold increase in circulating oxytocin in cattle. Physiological Genomics 43, 467–478.

De Pergola, G., 2000. The adipose tissue metabolism: role of testosterone and dehydroepiandrosterone. International Journal of Obesity and Related Metabolic Disorders 24 (Suppl. 2), S59–S63.

Edgar, R., Domrachev, M., Lash, A.E., 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Research 30, 207–210. Enns, D.L., Tiidus, P.M., 2010. The influence of Estrogen on skeletal muscle: sex matters. Sports Medicine 40, 41–58.

Giorgino, F., Smith, R.J., 1995. Dexamethasone enhances insulin-like growth factor-I effects on skeletal muscle cell proliferation. Role of specific intracellular signaling pathways. Journal of Clinical Investigation 96, 1473–1483.

Gray, L.E., Wilson, V.S., Stoker, T., Lambright, C., Furr, J., Noriega, N., et al., 2006. Adverse effects of environmental anti androgens and androgens on reproductive development in mammals. International Journal of Andrology 29, 96–104.

Griffin, C.A., Kafadar, K.A., Pavlath, G.K., 2009. MOR23 promotes muscle regeneration and regulates cell adhesion and migration. Developmental Cell 17, 649–661.

Gu, G., Varoqueaux, F., Simerly, R.B., 1999. Hormonal regulation of glutamate receptor gene expression in the anteroventral periventricular nucleus of the hypothalamus. The Journal of Neuroscience 19, 3213–3222.

Heisler, L.E., Evangelou, A., Lew, A.M., Trachtenberg, J., Elsholtz, H.P., Brown, T.J., 1997. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Molecular and Cellular Endocrinology 126, 59–73.

Henricks, D.M., Edwards, R.L., Champe, K.A., Gettys, T.W., Skelley, G.C., Jr., Gimenez, T., 1982. Trenbolone, estradiol-17 beta and estrone levels in plasma and tissues and live weight gains of heifers implanted with trenbolone acetate. Journal of Animal Science 55, 1048–1056.

Henricks, D.M., Brandt, R.T., Jr., Titgemeyer, E.C., Milton, C.T., 1997. Serum concentrations of trenbolone-17 beta and estradiol-17 beta and performance of heifers treated with trenbolone acetate, melengestrol acetate, or estradiol-17 beta. Journal of Animal Science 75, 2627–2633.

Holmäng, A., 1993. Metabolic effects of steroid hormones in skeletal muscle. BAM 3, 133–141. Hotchkiss, A.K., Furr, K., Makynen, E.A., Ankley, G.T., Gray, L.E., 2007. In utero exposure to the environmental androgen trenbolone masculinizes female Sprague-Dawley rats. Toxicology Letters 174, 31–41.

Hu, G., Lee, H., Price, S.M., Shen, M.M., Abate-Shen, C., 2001. Msx homeobox genes inhibit differentiation through upregulation of cyclin D1. Development (Cambridge, England) 128, 2373–2384.

Khandelwal, P., Liu, S., Sullivan, D.A., 2012. Androgen regulation of gene expression in human meibomian gland and conjunctival epithelial cells. Molecular Vision 18, 1055–1067.

Lancova, K., Dip, R., Antignac, J.P., Le Bizec, B., Elliott, C., Naegeli, H., 2011. Detection of hazardous food contaminants by transcriptomics fingerprinting. Trends in Analytical Chemistry 30, 181–191.

Lange, I.G., Daxenberger, A.,Meyer, H.H., 2001. Hormone contents in peripheral tissues after correct and off-label use of growth promoting hormones in cattle: effect of the implant preparations Filaplix-H, Raglo, Synovex-H and Synovex Plus. APMIS: Acta Pathologica, Microbiologica, et Immunologica Scandinavica 109, 53–65.

Maher, A.C., Akhtar, M., Tarnopolsky, M.A., 2010. Men supplemented with 17betaestradiol have increased beta-oxidation capacity in skeletal muscle. Physiological Genomics 42, 342–347.

Maxwell, M.A., Cleasby, M.E., Harding, A., Stark, A., Cooney, G.J., Muscat, G.E., 2005. Nur77 regulates lipolysis in skeletal muscle cells. Evidence for cross-talk between the beta-adrenergic and an orphan nuclear hormone receptor pathway. The Journal of Biological Chemistry 280, 12573–12584.

McKinsey, T.A., Zhang, C.L., Olson, E.N., 2000. Activation of the myocyte enhancer factor-2 transcription factor by calcium/.calmodulin-dependent protein kinasestimulated binding of 14-3-3 to histone deacetylase 5. Proceedings of the National Academy of Sciences 97, 14400–14405.

Middelbos, I.S., Vester, B.M., Karr-Lilienthal, L.K., Schook, L.B., Swanson, K.S., 2009. Age and diet affect gene expression profile in canine skeletal muscle. PLoS ONE 4, e4481.

Mooradian, A.D., Morley, J.E., Korenman, S.G., 1987. Biological actions of androgens. Endocrine Reviews 8, 1–28. Moore-Carrasco, R., García-Martínez, C., Busquets, S., Ametller, E., Barreiro, E., López-Soriano, F.J., et al., 2006. The AP-1/CJUN signaling cascade is involved in muscle differentiation: implications in muscle wasting during cancer cachexia. FEBS Letters 580, 691–696.

Moreno-Sánchez, N., Rueda, J., Reverter, A., Carabaño, M.J., Díaz, C., 2012. Muscle-specific gene expression is underscored by differential stressor responses and coexpression changes. Functional and Integrative Genomics 12, 93– 103.

Murray, J., Huss, J.M., 2011. Estrogen-related receptor? regulates skeletal myocyte differentiation via modulation of the ERK MAP kinase pathway. American Journal of Physiology. Cell Physiology 301, C630–C645.

Nebbia, C., Urbani, A., Carletti, M., Gardini, G., Balbo, A., Bertarelli, D., et al., 2011. Novel strategies for tracing the exposure of meat cattle to illegal growth-promoters. The Veterinary Journal 189, 34–42.

Odore, R., Badino, P., Barbero, R., Cuniberti, B., Pagliasso, S., Girardi, C., et al., 2007. Regulation of b-adrenergic, glucocorticoid and androgen receptors induced by repeated exposure to growth promoters in male veal calves. Research in Veterinary Science 83, 227–233.

Panet-Raymond, V., Gottlieb, B., Beitel, L.K., Pinsky, L., Trifiro, M.A., 2000. Interactions between androgen and estrogen receptors and the effects on their transactivational properties. Molecular and Cellular Endocrinology 167, 139– 150.

Pearson, G., Robinson, F., Gibson, T.B., Xu, B., Karandikar, M., Berman, K., et al., 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocrine Reviews 22, 153–183.

Pegolo, S., Gallina, G., Montesissa, C., Capolongo, F., Ferraresso, S., Pellizzari, C., et al., 2012. Transcriptomic markers meet the real world: finding diagnostic signatures of corticosteroid treatment in commercial beef samples. BMC Veterinary Research 30 (8), 205.

Pinel, G.,Weigel, S., Antignac, J.P., Mooney, M.H., Elliott, C., Nielen, M.W.F., et al., 2010. Targeted and untargeted profiling of biological fluids to screen for anabolic practices in cattle. Trends in Analytical Chemistry 29, 1269–1280.

Pradeep, P.K., Li, X., Peegel, H., Menon, K.M., 2002. Dihydrotestosterone inhibits granulosa cell proliferation by decreasing the cyclin D2 mRNA expression and cell cycle arrest at G1 phase. Endocrinology 143, 2930–2935.

Riedmaier, I., Becker, C., Pfaffl,M.W., Meyer, H.H., 2009. The use of omic technologies for biomarker development to trace functions of anabolic agents. The Journal of Chromatography A 1216, 8192–8199. Riedmaier, I., Benes, V., Blake, J., Bretschneider, N., Zinser, C., Becker, C., et al., 2012. RNA-sequencing as useful screening tool in the combat against the misuse of anabolic agents. Analytical Chemistry 84, 6863–6868.

Rijk, J.C., Peijnenburg, A.A., Hendriksen, P.J., Van Hende, J.M., Groot, M.J., Nielen, M.V., 2010. Feasibility of a liver transcriptomics approach to assess bovine treatment with the prohormonedehydroepiandrosterone (DHEA). BMC Veterinary Research 16 (6), 44.

Rodríguez, Y., Almeida, T.A., Valladares, F., Báez, D., Montes de Oca, F., García, C., et al., 2010. Neurotensin and neurotensin receptor 1 expression in humanmyometrium and uterine leiomyomas. Biology of Reproduction 83, 641–647.

Ruby, M.A., Goldenson, B., Orasanu, G., Johnston, T.P., Plutzky, J., Krauss, R.M., 2010. VLDL hydrolysis by LPL activates PPAR-α through generation of unbound fatty acids. The Journal of Lipid Research 51, 2275–2281.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., et al., 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34, 374–378.

Saeed, A.I., Bhagabati, N.K., Braisted, J.C., Liang,W., Sharov, V., Howe, E.A., et al., 2006. TM4 microarray software suite. Methods in Enzymology 411, 134–193. Singh, R., Bhasin, S., Braga, M., Artaza, J.N., Pervin, S., Taylor, W.E., et al., 2009. Regulation ofmyogenic differentiation by androgens: cross talk between androgen receptor/beta-catenin and follistatin/transforming growth factor-beta signaling pathways. Endocrinology 150, 1259–1268.

Sinha-Hikim, I., Roth, S.M., Lee, M.I., Bhasin, S., 2003. Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. American Journal of Physiology. Endocrinology and Metabolism 285, E197–E205.

Sinha-Hikim, I., Taylor, W.E., Gonzalez-Cadavid, N.F., Zheng, W., Bhasin, S., 2004. Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment. The Journal of Clinical Endocrinology and Metabolism 89, 5245–5255.

Stella, R., Biancotto, G., Krogh, M., Angeletti, R., Pozza, G., Sorgato, M.C., et al., 2011. Protein expression changes in skeletal muscle in response to growth promoter abuse in beef cattle. Journal of Proteome Research 10, 2744–2757.

Sun, L., Ma, K.,Wang, H., Xiao, F., Gao, Y., Zhang,W., et al., 2007. JAK1-STAT1-STAT3, a key pathway promoting proliferation and preventing premature differentiation of myoblasts. The Journal of Cell Biologyy 179, 129–138. Svensson, J., Movérare-Skrtic, S., Windahl, S., Swanson, C., Sjögren, K., 2010. Stimulation of both estrogen and androgen receptors maintains skeletal muscle mass in gonadectomized male mice but mainly via different pathways. Journal of Molecular Endocrinology 45, 45–57.

Tang, Z., Ow, G.S., Thiery, J.P., Ivshina, A.V., Kuznetsov, V.A., 2013. Meta-analysis of transcriptome reveals let-7b as an unfavorable prognostic biomarker and predicts molecular and clinical sub-classes in high-grade serous ovarian carcinoma. International Journal of Cancer doi:10.1002/ijc.28371.

Tibshirani, R., Hastie, T., Narasimhan, B., Chu, G., 2002. Diagnosis of multiple cancer types by shrunken centroids of gene expression. PNAS USA 99, 6567–6572. Vasconsuelo, A., Pronsato, L., Ronda, A.C., Boland, R., Milanesi, L., 2011. Role of 17beta-estradiol and testosterone in apoptosis. Steroids 76, 1223–1231. Vogt, K., Oehrle, K.L., 1977. Archiv für Lebensmittelhygiene 28, 44.

Yeap, B.B., Krueger, R.G., Leedman, P.J., 1999. Differential posttranscriptional regulation of androgen receptor gene expression by androgen in prostate and breast cancer cells. Endocrinology 140, 3282–3291.

Zhou, F., Bouillard, B., Pharaboz-Joly, M.O., Andre, J., 1989. Non-classical antiestrogenic actions of dexamethasone in variant MCF-7 human breast cancer cells in culture. Mol. Cell. Endocrinology 66, 189–197.