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RNA-Sequencing as Useful Screening Tool in the Combat against the Misuse of Anabolic Agents

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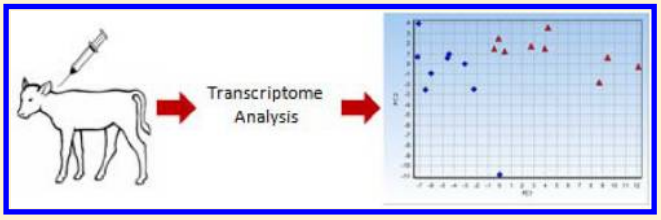
Supporting Information

ABSTRACT: The abuse of anabolic substances in animal husbandry is forbidden within the EU and well controlled by detecting substance residues in different matrices. The application of newly designed drugs or substance cocktails represents big problems. Therefore developing sensitive test methods is important. The analysis of physiological changes caused by the use of anabolic agents on the molecular level, for example, by quantifying gene expression response, is a new approach to develop such screening methods. A novel technology for holistic gene expression analysis is RNA sequencing. In this study, the potential of this high-throughput method for the identification of biomarkers was evaluated. The effect of trenbolone acetate plus estradiol on gene expression in liver from Nguni heifers was analyzed with RNA sequencing. The expression of 40 selected candidate genes was verified via RT-qPCR, whereby 20 of these genes were significantly regulated. To extract the intended information from these regulated genes, biostatistical tools for pattern recognition were applied and resulted in a clear separation of the treatment groups. Those candidate genes could be verified in boars and in calves treated with anabolic substances. These results show the potential of RNA sequencing to screen for biomarker candidates to detect the abuse of anabolics. The verification of these biomarkers in boars and calves leads to the assumption that gene expression biomarkers are independent of breed or even species and that biomarkers, identified in farm animals could also act as potential biomarker candidates to detect the abuse of anabolic substances in human sports.



Growth promoting agents, like steroid hormones or β-agonists, are beneficial for increasing muscle mass and decomposition of lipid tissue. In animal husbandry, the use of growth promoting agents, mainly, steroid hormones, growth hormone or β-agonists is economically also very attractive.[1,2] In some countries, among others the USA, Canada, and South Africa, the application of specific anabolic agents in animal husbandry is licensed, but because of proven side effects for the consumer, the use of all growth promoting agents is forbidden in the EU (Directive 96/22/EC). Within strict control programs, the misuse of these substances is monitored by detecting residues of all known growth promoters and performance enhancers using immuno assays or chromatography combined with mass spectrometry.[3−5] Newly designed compounds with unknown structure cannot be detected with these established methods and exogenous applied natural hormones like estradiol can hardly be distinguished from endogenous substances. Another problem is the application of hormone cocktails consisting of multiple substances, resulting in residues at a concentration below the detection limit of the usual techniques.[6] Therefore, the development of new, very sensitive detection methods is necessary.

Regardless of which growth promoter is used, the desired effect is the same: increase of muscle mass and decrease of fat tissue. This produces changes in the physiological state of the individual, which are measurable on the molecular level. Detection of these effects is a highly discussed way in the combat against the misuse of anabolic substances.[7−9] Molecular detection of physiological alterations can be performed on different levels, namely the transcriptome, the proteome or the metabolome. Steroid hormones, the main substance group applied for growth promoting purpose, directly influence the transcription of hormone sensitive target genes. Their receptors act as transcription factors for certain genes upon binding of their ligand. A second group of growth promoters, β-agonists indirectly affect transcription by activating a G-protein coupled phosphorylation cascade.[8] Therefore, the analysis of the

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transcriptome for the detection of physiological changes caused by anabolic agents is a very promising way. Different publications are already available, dealing with the analysis of the expression of different genes to find gene expression biomarkers for the misuse of growth promoting agents in humans and animals. It can be distinguished between two strategies of analysis. Analysis can either be done in a holistic way by screening for the expression of a large number of genes in one experiment or by single assays analyzing a limited number of candidate genes, chosen by screening the literature for physiological changes caused by treatment.[8,9] Most published studies regarding changes in the transcriptome caused by the application of anabolic agents describe the analysis of the expression of a limited number of genes by RT-qPCR in different tissues.[10−19] There are also studies published applying the "untargeted approach" by screening for a large number of expressed genes using cDNA microarray technology.[20,21] Single gene expression biomarker detection for the application of anabolic substances seems to be difficult, but the identification of a group or pattern of regulated factors is very promising.

Biostatistical tools for dimensionality reduction and pattern recognition were applied in some studies to extract the needed information from a set of regulated genes. Thereby, principal components analysis (PCA) and hierarchical cluster analysis (HCA) have shown to be helpful tools.[22] By analyzing a pattern of significantly regulated genes with these methods, a separation between treated and untreated individuals could be visualized, but in most cases the groups were not separated with high confidence, indicating that the gene expression biomarker candidates are not sufficient for treatment screening yet.[15−18,21] Screening for a huge number of genes in one experiment seems to be very promising, because this approach is "hypothesis free" and expression changes of genes that would not be expected can also be identified. Microarray technology is up to now the "gold standard" to screen for gene expression changes caused by treatment, disease etc in a holistic way. The development of high throughput sequencing technologies enabled the establishment of a new method for gene expression analysis, called RNA-Sequencing (RNA-Seq). This method allows the detection of all expressed mRNAs, being able to detect ″one single RNA molecule″ and is thereby nearly as sensitive as RT-qPCR.[23] Compared with the microarray method, RNA-Seq has no upper limit of detection, shows a higher dynamic range of expression levels and has nearly no background signal.[23] Another advantage of this very new technology is the possibility of de novo detection enabling the identification of new mRNAs or new splice variants of expressed genes.[24]

The aim of this study was to test the potential of RNA-Seq technology for the identification of gene expression biomarkers for the use of anabolic agents in farm animals.

Therefore liver samples from "Nguni" heifers treated with a combination of trenbolone acetate plus estradiol and from untreated heifers were analyzed using RNA-Seq. Identified biomarker candidates were verified in liver samples from "crossbreed" boars (German Landrace x Pietrain) also treated with a combination of trenbolone acetate plus estradiol and in "Holstein Friesian" calves treated with a combination of hormone esters.

EXPERIMENTAL SECTION

Animal Experiments. Liver samples from three independent animal studies were analyzed. In animal experiment 1, Nguni heifers were treated with a combination of trenbolone acetate plus estradiol. In animal experiment 2, crossbreed boars were also treated with a combination of trenbolone acetate plus estradiol, and in animal experiment 3, calves were treated with a combination of estradiolbenzoate, testosteronedecanoate, and testosteronecypionate via pour on. In all three animal experiments, one group of animals remained untreated, serving as control animals. A detailed description of the animal experiments is given in Supporting Information Text 1.

Gene Expression Analysis. The workflow for producing reliable gene expression data has been defined by different researchers who published the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments", also called the MIQE guidelines.[25] Gene expression analysis in the experiments of this study were performed according to these guidelines.

RNA was extracted from liver samples using standard protocols and the quality of extracted RNA was determined using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). RNA-Sequencing was done with samples from two control animals and three treated animals from animal experiment 1 in a single-read, 76-base mode on a GAIIx Sequencer (Illumina, San Diego, USA) and RNA-Seq data were analyzed using the Genomatix Mining station (Genomatix Software GmbH, Munich, Germany) and the NGSAnalyzer (ElDorado 02−2010; Genomatix Software GmbH) utilizing the ElDorado database. Results obtained from the RNA-Sequencing experiment were selected due to the following parameters: significancy of regulation ($p > 0.01$), normalized expression value >0.01 and treatment/control ratio lower than 0.6 or higher than 5. A total of 40 genes were chosen in this way and validated in all 9 samples per group using single assay real-time RT-qPCR.

RT-qPCR data analysis was done using relative quantification method described by Livak and Schmittgen.[26] Reference genes for data normalization were chosen using GenEx Pro Software Version 5 (MultiD Analyses AB, Gothenburg, Sweden). The geometric mean of two selected reference genes (Ubiquitin, Histon H3) was used as reference gene index (RGI) for normalization.

Significantly regulated genes from animal experiment 1 were validated in biological liver samples from animal experiment 2 and 3 using RT-qPCR. To visualize the multivariate response of the selected classifiers to the treatment, significantly regulated genes from all three animal experiments were analyzed via principal components analysis (PCA) and hierarchical cluster analysis (HCA) using *GenEx*, version 5 (MultiD Analyses AB).

A description of all protocols and technical details used in gene expression analysis is given in Supporting Information Text 2.

RESULTS

RNA Integrity. The RNA integrity was measured using the lab-on-a-chip technology of the 2100 Bioanalyzer (Agilent Technologies). The RIN value of the liver samples obtained in Nguni heifers was 8.34 ± 0.36 (mean ± SD). The RIN value of the liver samples obtained in boars was 7.55 ± 0.33 (mean ± SD). RNA from liver samples extracted from calves showed RIN values of 7.6 ± 0.67(mean ± SD). RNA quality in all three animal trials showed high RIN numbers, indicating intact RNA.

RNA-Sequencing. The number of 76bp sequences was 23.429.884 for control animal 5, 22.352.193 for control animal 8, 25.350.551 for treated animal 3, 25.310.479 for treated

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animal 5 and 17.810.394 for treated animal 8. Mapping to the genome resulted in 14.146.526 (control animal 5), 11.642.389 (control animal 8), 12.980.903 (treated animal 3), 16.550.838 (treated animal 5), and 10.933.861 (treated animal 8) unique hits, respectively. Gene expression analysis resulted in 9331 significantly regulated transcripts between control and treatment group.

RT-qPCR. A total of 40 candidate genes, significantly regulated in the RNA-Seq experiment were chosen to be validated by RT-qPCR in all 18 liver samples. Twenty genes were confirmed to be significantly regulated. Herein, 9 genes were significantly down-regulated and 11 genes were significantly up-regulated. The direction of regulation was similar to the RNA-Seq results except of EPYC which was down-regulated in the RNA-Seq experiment and up-regulated in the quantification via RT-qPCR. A summary of all verified genes, including level of significance (p-value) and x-fold regulations of RNA-Seq and RT-qPCR is given in Table 1.

PCA and HCA were applied to test, if these regulated genes could be potential biomarkers to distinguish between treated and untreated animals. Figure 1 shows the PCA and HCA results obtained by analyzing the 20 significantly regulated genes. With both methods, a clear separation between the different groups could be obtained and in the PCA, groups can be separated by drawing ellipses.

After the potential of these 20 genes to act as biomarkers in animal trial 1 could be shown, their expression was also quantified in samples obtained from boars (animal experiment 2) and calves (animal experiment 3). Fourteen genes could successfully be quantified in boars, whereas 4 of these genes showed a significant regulation between control and treatment group (table 2). PCA and HCA analysis was done implicating the results of all 14 quantifiable genes. Figure 2 shows, that a clear separation between treated and untreated boars is visible by applying both statistical methods and in the PCA, groups can also be separated by drawing ellipses. In liver samples obtained in the pour on trial on calves, only one gene was significantly regulated in the group treated once with the hormone mix and three genes showed significant regulation in the three times treated group, also a fourth gene showed a trend for regulation (p = 0.091). x-Fold regulations and corresponding p-values are listed in table 2. PCA and HCA were performed with the four regulated genes (figure 3). In the PCA analysis the three times treated group separates from the animals of the other groups, but there is also an incomplete separation between control animals and calves treated with the hormone mix only once. In the HCA analysis the three times treated animals are arranged at the bottom of the dendogram, whereas the other animals are randomly mixed up.

Table 1. List of Genes Verified by RT-qPCR x-Fold Regulation of the RNA-Seq Experiment and of the Validation by RT-qPCR[a]

gen	x-fold regulation RNA-Seq (n = 2/3)	x-fold regulation PCR (n = 9)	p-value PCR (n = 9)
SERPINI2	26.18	**82.36**	**0.01**
AK3L1	6.62	**13.66**	**0.003**
TNC	8.06	**8.87**	**0.000**
HOPX	10.77	**8.18**	**0.038**
EPYC	0.12	**6.69**	**0.036**
CUX2	26.76	**6.33**	**0.00**
HBB	7.04	**5.57**	**0.023**
CLSTN2	65.00	5.15	0.510
CLDN4	6.08	**4.52**	**0.007**
GDPD1	8.39	**4.18**	**0.001**
ITGAD	23.07	4.01	0.670
SECTM1	6.42	**3.66**	**0.037**
PRODH2	7.79	**3.59**	**0.000**
FABP3	7.60	3.31	0.432
STYK1	15.39	2.49	0.943
ALAS2	11.16	2.29	0.391
FAP	0.11	2.04	0.700
SLC13A2	0.23	1.92	0.578
ADRB2	2.58	1.81	0.745
SLC6A1	11.00	1.58	0.828
GIMAP4	0.09	1.45	0.675
RUNDC3A	13.26	1.40	0.922
CPA3	0.10	1.17	0.979
CYP17	14.50	1.08	0.549
GPNMB	0.22	0.99	0.437
VNN1	0.20	0.80	0.534
CHRM2	0.09	0.78	0.430
RETN	0.10	0.75	0.550
MSMB	0.04	0.72	0.280
CXCL9	0.09	0.71	0.424
GSTM1	0.22	**0.57**	**0.022**
MX1	0.44	0.56	0.244
ISG15	0.33	**0.54**	**0.058**
CXCL10	0.13	**0.53**	**0.028**
MX2	0.28	**0.47**	**0.019**
SOD3	0.16	**0.40**	**0.007**
CYP2E1	0.02	**0.38**	**0.015**
FADS2	0.09	**0.37**	**0.004**
PON1	0.15	**0.28**	**0.001**
APOA4	0.28	**0.19**	**0.008**

[a]Significant regulations in the RT-qPCR experiment are marked in bold.

DISCUSSION

The use of omic technologies is a highly discussed new approach for the identification of the abuse of anabolic agents in farm animals.[8] With these technologies physiological changes caused by the application of such substances can be detected on the molecular level. One promising way is the detection of gene expression changes, because the main substances used for anabolic purpose - steroid hormones and β-agonists - are known to directly influence the expression of specific genes.[8] Up to now, gene expression changes were quantified either with RT-qPCR, analyzing only a defined number of mRNAs or by gene expression microarrays, screening for the expression of a high number of genes in one experiment.[12−21] High-throughput sequencing has enabled the development of a new technology for quantification of gene expression, namely RNA-Seq which is more sensitive than microarray technology and enables the detection of higher expression differences.[23]

The aim of this study was to test the potential of RNA-Seq technology to screen for potential gene expression biomarkers to detect the illegal use of anabolic agents in farm animals.

Gene expression analysis following the targeted approach, analyzing the expression of a panel of genes chosen by literature research was already done in the liver samples analyzed by RNA-Seq in this study.[16] Thereby, 34 candidate genes belonging to different functional groups, like receptors, endocrine factors, protein metabolism, transcription factors, immune factors, factors involved in cholesterol metabolism,

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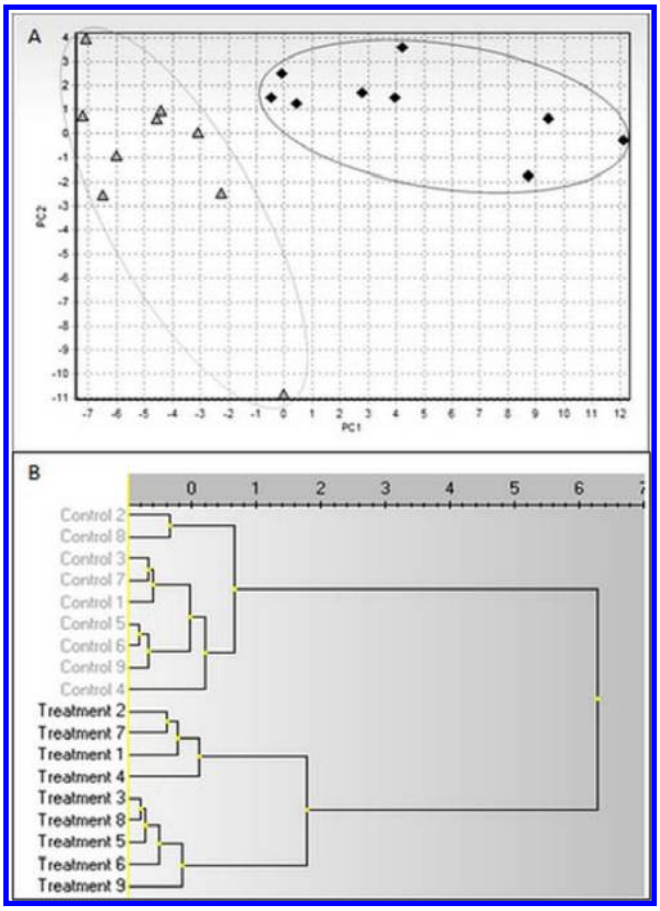

Figure 1. Principal components analysis (A) and hierarchical cluster analysis (B) for the 20 regulated genes in Nguni heifers. Animals of the control group are represented by gray triangles and animals of the treatment group are represented by black diamonds.

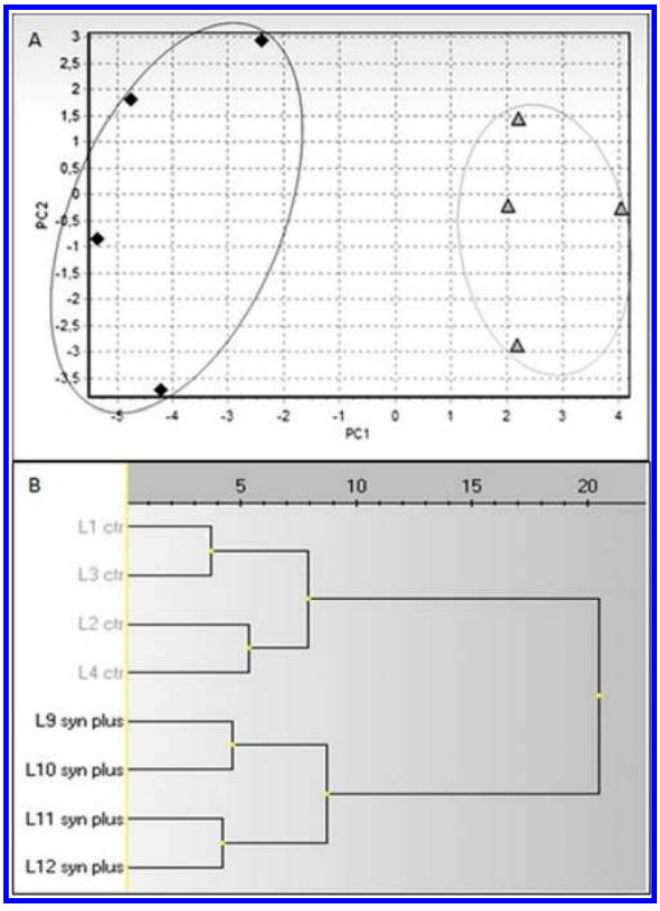

Figure 2. Principal components analysis (A) and hierarchical cluster analysis (B) for the 14 measurable genes in boars. Animals of the control group are represented by gray triangles and animals of the treatment group are represented by black diamonds.

Table 2. List of Candiate Genes Verified in Animal Experiment 2 (Boars) and 3 (Calves) *x*-Fold Regulation of the RNA-Seq Experiment and *x*-Fold Regulation Plus Level of Significance of the Validation by RT-qPCR in All Three Animal Experiments

gene	*x*-fold NGS	*x*-fold PCR Nguni heifers	*p*-value PCR Nguni heifers	*x*-fold PCR boars	*p*-value PCR boars	*x*-fold PCR calves treatment 1×	*p*-value PCR calves treatment 1×	*x*-fold PCR calves treatment 3×	*p*-value PCR calves treatment 3×
CYP2E1	**0.02**	**0.38**	**0.015**	**0.04**	**0.003**	1.88	0.192	1.58	0.113
FADS2	**0.09**	**0.37**	**0.004**	**0.18**	**0.012**	1.25	0.369	1.08	0.916
CXCL10	**0.13**	**0.53**	**0.028**	0.54	0.120				
PON1	**0.15**	**0.28**	**0.001**	0.57	0.094	1.16	0.750	**0.51**	**0.047**
SOD3	**0.16**	**0.40**	**0.007**			1.68	0.264	**0.54**	**0.041**
GSTM1	**0.22**	**0.57**	**0.022**			1.18	0.734	0.81	0.284
MX2	**0.28**	**0.47**	**0.019**	1.25	0.871	0.92	0.770	0.73	0.322
APOA4	**0.28**	**0.19**	**0.008**	0.34	0.075	3.40	0.115	4.99	0.954
ISG15	**0.33**	**0.54**	**0.058**	2.23	0.256	0.53	0.169	0.84	0.611
CLDN4	**6.08**	**4.52**	**0.007**	2.25	0.306	**2.27**	**0.006**	**3.02**	**0.014**
SECTM1	**6.42**	**3.66**	**0.037**			1.64	0.448	1.09	0.787
AK3L1	**6.62**	**13.66**	**0.003**			0.85	0.483	1.33	0.552
HBB	**7.04**	**5.57**	**0.023**	0.50	0.077	1.33	0.804	1.64	0.668
PRODH2	**7.79**	**3.59**	**0.000**	**1.30**	**0.047**	1.22	0.571	1.20	0.745
TNC	**8.06**	**8.87**	**0.000**	1.73	0.447	1.79	0.331	1.09	0.802
GDPD1	**8.39**	**4.18**	**0.001**			0.72	0.271	1.25	0.837
HOPX	**10.77**	**8.18**	**0.038**	2.01	0.122	1.61	0.761	1.08	0.558
SERPINI2	**26.18**	**82.36**	**0.012**	1.85	0.524	0.62	0.513	0.37	0.159
CUX2	**26.76**	**6.33**	**0.000**	**5.04**	**0.056**	1.21	0.971	0.65	0.091
EPYC	**0.12**	**6.69**	**0.036**			1.32	0.841	0.77	0.381

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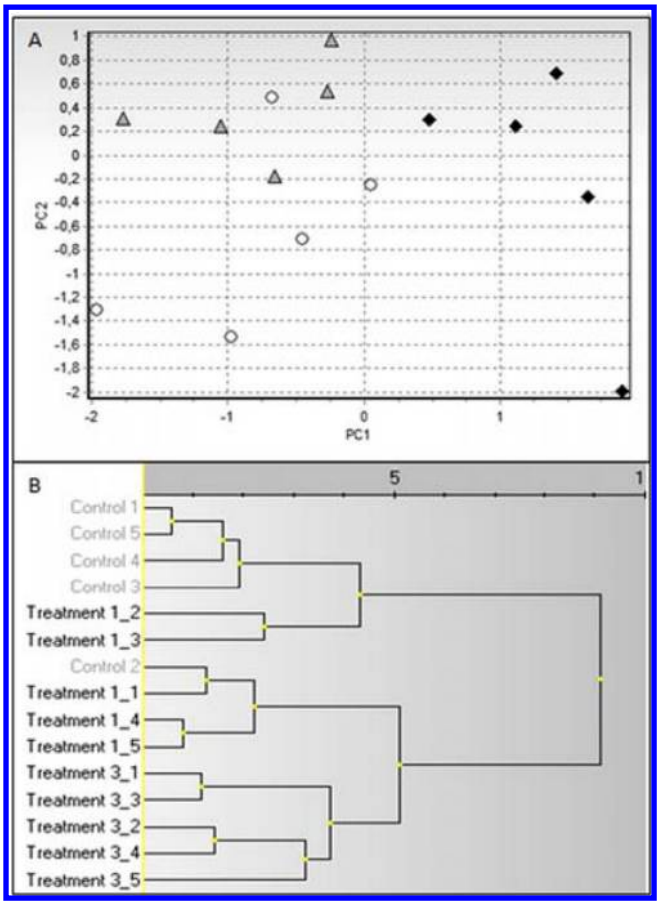

Figure 3. Principal components analysis (A) and hierarchical cluster analysis (B) for the 4 regulated genes in calves treated via pour on. Animals of the control group are represented by gray triangles, animals of the group treated once with the hormone mix are represented by white circles and animals of the group treated three times with the hormones are represented by black diamonds.

factors involved in steroid metabolism and factors involved in lipid metabolism were analyzed by RT-qPCR. Eleven genes were identified to be significantly regulated by the implantation of Revalor H (Intervet), whereat fold regulation values ranged between 0.27 for IGFBP-2 and 1.89 for IGF-1. Principal components analysis (PCA) of regulated genes showed a separation between treated and untreated animals, but separation was incomplete in the mean area.[16] Expression analysis of 40 candidate genes chosen by RNA-Seq resulted in 20 significantly regulated genes with fold regulation values from 0.19 for APOA4 up to 82.36 for SerpinI2. This shows that hypothesis free gene expression screening by RNA-Seq results in more significant and higher regulated genes than by choosing candidates by literature research. This technology enables quantification of changes in the expression of non expected genes or new splice variants and the influence of treatment with anabolic agents on pathways that are not known to be influenced yet can also be detected.[23]

Analysis of these 20 biomarker candidates with PCA and HCA visualized a clear separation between treated and untreated animals indicating that these 20 genes could act as first biomarkers for identifying heifers treated with Revalor H. To verify these candidate genes, their expression was quantified in liver samples from boars treated with Synovex plus, also an implant containing a combination of trenbolone acetate plus estradiol and in calves treated with a mix of hormone esters via pour on. In boars, only 4 genes were significantly regulated with expression values from 0.04 for CYP2E1 to 5.04 for CUX2 but analysis via PCA and HCA using all measured genes also resulted in a complete separation between treated and untreated animals. Pour on treatment in calves resulted in the regulation of one or four genes out of 20 candidates, respectively, with expression values of 2.27 (treatment once) and between 0.51 and 3.02 (treatment three times). Analysis of these regulated genes with clustering methods resulted in a separation of the three times treated group from the other two groups, which did not separate completely from each other. The anabolic effect of the pour on treatment monitored by differences in weight gain of the treatment groups compared to the control animals was significant in the three times treated group but not in the animals treated with the hormone mix only once.[27] This indicates that only one treatment via pour on did not have a significant anabolic effect and that therefore the control and one time treated group do not separate using the clustering methods.

The results obtained in Nguni heifers and boars indicate that identified regulated genes represent first biomarker candidates for the detection of treatment with a combination of trenbolone acetate plus estradiol in farm animals. In calves treated with a hormone cocktail containing androgens and estrogens, four out of these 20 candidates could be verified as biomarker candidates, leading to the assumption that these genes also can act as first biomarker candidates for the detection of treatment with anabolic combinations of androgens plus estrogens but that the list of biomarker candidates have to be completed in this respect. Another important fact that could be shown by analyzing samples from different species, breed, sex, reproductive state, and age is that gene expression biomarkers seem to be independent of these parameters.

CONCLUSIONS

This study demonstrates that RNA-Seq is a very potent method to screen for highly regulated genes that can act as biomarker candidates for detecting the misuse of anabolic agents in farm animals. With this method, 20 candidate genes could be identified enabling separation of heifers treated with trenbolone acetate plus estradiol from untreated individuals by analyzing their expression with clustering methods like PCA or HCA. These candidate genes could be verified in boars also treated with a combination of trenbolone acetate plus estradiol, indicating that these genes can act as first biomarkers for treatment with the combination of these substances. Results, obtained in calves treated via pour on show that these biomarkers could also act as first candidates for treatment with an anabolic combination of androgens plus estrogens.

For the verification of these genes and for the identification of new potential biomarker candidates, more trials with other anabolic substances and different species are necessary. Another important point will be the collection of a high number of untreated samples to cover a broad range of influencing factors like age, immune status, housing conditions, etc. The evaluation of gene expression changes in other matrices, for example, blood which can be taken in a noninvasive way from the living individual, will be of importance.

The fact that biomarkers identified in cattle and calves could be verified in pigs leads to the hypothesis that gene expression biomarkers are independent of species and that therefore genes whose expression is changed in animals can also act as potential

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biomarkers for the detection of the misuse of anabolic agents in human sports.

■ ASSOCIATED CONTENT

Supporting Information

Additional material as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

⊥Deceased April 13, 2012.

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