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Development of a Uniform Biomarker Signature in Calves Heart and Lung to Detect the Abuse of Different Anabolic Substances

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Abstract

The use of growth-promoting agents in food-producing animals is economically very attractive for farmers. Due to proven side effects of substance residues for the consumer, the administration of growth-promoting agents is forbidden in the EU and this ban is well controlled. But, new designed xenobiotic drugs and unknown application regimes like the administration of hormone cocktails are still difficult to detect. In the search for alternative detection methods, the development of molecular biomarkers tracing the physiological effects of the applied substances has come into focus.

In this context, the identification of transcriptional biomarkers has already proven to be successful. Previously, a first biomarker signature for the treatment of clenbuterol as well as progesterone plus estradiol benzoate in calves liver could be identified on mRNA level. Within the current study, gene expression in lung and heart tissue from the same animal trial was analyzed in order to identify additional mRNA biomarkers.

Candidate biomarkers were chosen by literature search for already known effects of the applied drugs on the respective tissue and corresponding mRNA expression levels were quantified by RT-qPCR.

Dynamic Principal Components Analysis (PCA) was used in order to identify an mRNA biomarker signature that enables the separation of treated from untreated animals. In heart tissue, a signature of 22 expressed genes could be identified for steroid treatment and a signature of 12 genes for clenbuterol treatment. In lung tissue, a signature of 4 genes was identified for steroid treatment and a signature of 8 genes for clenbuterol treatment. As the separation of the treatment groups was not optimal in both tissues, integrative data analysis was performed, using heart and lung expression results. Thereby a signature of 17 genes showed to be the best for separation of control animals from steroid-treated animals and a signature of 9 genes for clenbuterol-treated animals. Additionally, a signature of 23 genes could be identified, that enabled separation of untreated from treated animals, independent of the applied substance. To prove the predictive power of built discriminative models, Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) was applied, displaying results in high quality.

The results of this study indicate the high potential of developing transcriptional biomarkers for the detection of the illegal use of growth promoting agents in livestock animals.

Keywords: Steroids; Clenbuterol; Transcriptome; Biomarker signature; Lung

Introduction

Anabolic agents like steroid hormones or β-agonists are used in food-producing animals in order to increase muscle mass and reduce fat content [1,2]. The application of specific anabolic agents in animal husbandry is allowed and common in different countries, e.g. USA, Canada, South Africa, etc. Due to proven side effects of substance residues for the consumer, the use of all growth promoting agents is forbidden within the European Union (EU) (Directives 96/22/EC, 96/23 EC, 2008/97/EC). To monitor this ban, routine controls are performed in animal samples from stables and slaughterhouses. Within this control system, substance residues are detected in different organs and matrices, like blood, urine, hair, eyeballs and other tissues using immunoassays or chromatography methods combined with mass spectrometry [3]. But new xenobiotic substances or treatment regimens still present a problem for the control laboratories. For example, the distinction between endogenous hormones and natural hormones that are applied exogenously is methodically demanding. Another challenge is the detection of hormone cocktails, where a mixture of different growth promoters is applied, and each hormone in a concentration lower

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than the detection limit of the certified control methods [4]. For this reason, the search for alternative innovative detection methods is an important research field. Thereby, the approach of identifying molecular biomarkers is very promising [5,6]. This approach is based on the analysis of physiological changes caused by the treatment with growth promoting agents. The intended physiological effect is an increased muscle growth and a decreased fat content, regardless of the growth-promoting substance. Also other organs like liver, kidney, heart, lung etc. are physiologically influenced as well by growth-promoting agents and therefore these are further potential target tissues for molecular biomarker screening. There are different levels on which such biomarkers are detectable, namely the transcriptome, the proteome or the metabolome. Up to now, identified biomarkers are still substance specific [3,7-10]. In order to be applicable a screening method for treated animals, the ideal biomarker pattern should be independent of the applied anabolic substances, or other conditions, like breed, age, gender or even species.

We could already identify a first biomarker signature based on changes on the level of the transcriptome in the liver of calves treated with a steroid implant or clenbuterol respectively, which enabled to distinguish untreated from treated animals, independent of the applied drug [11].

It is known, that the applied substances of this trial also had an influence on the physiology of heart and lung. Clenbuterol is bound to β-adrenergic receptors, which are also present in heart tissue [12]. Different studies have already shown that, β-agonists alter cardiovascular function, e.g. influencing heart rate, contractility and blood pressure [13]. An effect on cardiac muscle growth could also be shown [14,15]. Steroid hormones also have proven effects on heart tissue. Abuse of anabolic steroids seems to induce cardiac arrhythmia, hypertrophy, thrombogenesis or congestive heart failure [16].

Clenbuterol was originally designed for the treatment of asthma as a bronchospasmolytic agent. It mainly binds to β2-adrenergic receptors, which are present in the bronchial tubes and in lung tissue. It has a direct effect on smooth muscles in the lung [17]. In contrast, there is not much known about the effect of steroid hormones on lung tissue, yet.

As minimally one of the applied substances has a direct physiological influence on the heart or lung, the gene expression in those tissues was quantified, in order to identify additional biomarkers on the transcriptional level.

Material and Methods

Animal Experiment

21 male, 6 month old Holstein Friesian calves were separated into three equal groups. One group was untreated (control), the second group was treated with a combination of progesterone plus estradiol benzoate (steroid) and the last group was treated with clenbuterol. A detailed description of the animal trial is given in Riedmaier et al. 2014 [11].

Gene Expression Analysis

Gene expression analysis was performed according to the MIQE guidelines [18].

RNA was extracted from heart and lung samples using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer´s protocol. RNA purity was calculated using the $OD_{260/280}$ ratio. RNA quality was determined using the Eukaryotic total RNA Nano Assay on the 2100 Bioanalyzer (Agilent Technology, Palo Alto, USA).

For cDNA synthesis, constant amounts of 500 ng integer total RNA were reverse transcribed as already described before [19].

To analyze the expression of candidate genes, qPCR analysis was done using the iQ5 detection system (Bio-Rad, Munich, Germany) as already described before [11].

Candidate genes were chosen by screening the current literature for the effects of steroid hormones and clenbuterol on the respective organs and by analyzing different biochemical pathways, where the respective substance or factors that showed significant regulation are involved. Selected genes can be summarized in the functional groups of hormone receptors, transcription factors, proliferation factors, regulators of angiogenesis, apoptosis, blood pressure, protein, glucose & lipid metabolism, immune factors, oncogenes, structural proteins and different other factors. In total 80 genes were quantified in heart tissue and 90 genes were quantified in lung tissue, whereas 48 of those genes were quantified in both tissues. A detailed list of quantified genes and traits of all primer pairs is given in supplemental Tables 1 and 2.

The analysis of significantly regulated genes and multivariate data analysis was done as described before [11,20]. Dynamic PCA was performed with the normalized gene expression data from each tissue separately and in combination using GenEx version 6 (MultiD Analyses AB, Gothenburg, Sweden). Within this method, an ideal biomarker signature can be chosen using step by step exclusion of genes due to a declining p-value or a rising distance of regulation [11].

Additionally Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) was performed using SIMCA software (Umetrics, Umea, Sweden) in order to build statistical models. OPLS-DA is a well-known statistical method used in 'omics research' like genomics, proteomics or metabolomics [21,22]. OPLS removes undesired variability in complex data sets and can though be used in multivariate calibration and development of various filters. It separates non-correlated and correlated variation and decreases the total number of components used. OPLS-DA is performed to sharpen the separation between groups and to filter for variables carrying the class separation information [23]. Within the resulting scatter plots, horizontal direction gives information about inter-class variation and vertical direction shows variations within the classes. SIMCA software provides two parameters that give information about the quality of the OPLS-DA models. R2 (cum) describes how well the model fits the X data and thereby how well the model is able

to separate between the groups. The closer the value to 1, the better the model fits the X data. Q2 (cum) describes how well the generated model will predict new data. A value >0.5 indicates a good predictability of the designed model. In contrast to PCA analysis, groups have to be defined before OPLS-DA performance in order to find the best parameters to build a statistical model for group separation.

Results

RNA quantity and quality

The quantity and purity of the extracted total RNA were determined using the Nano Drop Photometer (Peqlab, Erlangen, Germany). Mean RNA concentration was 768 ± 63 ng/µL for heart tissue and 1380 ± 430 ng/µL for lung tissue, respectively. The $OD_{260/280}$ ratio is an indicator for RNA purity whereas a ratio >1.8 is considered as adequate for RT-qPCR experiments. Mean $OD_{260/280}$ was 2.14 ± 0.009 for heart tissue and 2.05 ± 0.01 for lung tissue, respectively.

RNA quality and integrity was determined using the Eukaryotic total RNA Nano Assay on the 2100 Bioanalyzer (Agilent Technologies). RNA Integrity Number (RIN) >7 can be considered as good quality RNA that is usable for RT-qPCR experiments. The mean RIN for the heart samples was 7.25 ± 0.24 and for the lung samples 8.2 ± 0.4, indicating intact RNA [24].

Influence of treatment on gene expression in heart

Both substance groups that were applied to the calves in this study are known to have a physiological influence on heart tissue.

In the steroid treated group, four genes were significantly up-regulated and 8 genes were significantly down-regulated. The Glucocorticoid Receptor α (GRα) was significantly down-regulated by 0.741 fold *(p=0.021).* In the group of proliferation and transcription factors, the Bone Morphogenetic Protein 2 Receptor (BMPR2) was significantly down-regulated by 0.792 fold *(p=0.033),* SMAD Family Member 2 (SMAD2) was significantly down-regulated by 0.727 fold *(p=0.016),* RB1 (Retinoblastoma-1) was significantly down-regulated by 0.840 fold *(p=0.034)* and NFκB (inflammatory factor nuclear factor of kappa light polypeptide gene) was significantly up-regulated by 1.482 fold *(p=0.031).* The angiogenesis regulators Vascular Endothelial Growth Factors Receptor 1 (FLT-1) and Angiopoietin-1 (ANGPT-1) were significantly down-regulated by 0.695 fold *(p=0.021)* or 0.606 fold *(p=0.016)* respectively. Calpastatin (CAST), a factor involved in protein metabolism showed a significant down-regulation by 0.769 fold *(p=0.033)* and Phospholipase C γ (PLCγ), a factor involved in lipid metabolism was significantly up-regulated by 1.454 fold *(p=0.059).* The Insulin Receptor β (IRβ) was significantly down-regulated by 0.777 fold *(p=0.011).* Two more factors, namely serpin peptidase inhibitor, clade E 2 (SerpinE2) and Protein Kinase C (PKC) were significantly up-regulated by 1.825 fold *(p=0.015)* or 1.399 fold *(p=0.051)* respectively.

In the clenbuterol-treated animals, three genes were shown to be significantly up-regulated and five genes were significantly down-regulated. The Estrogen Receptor α (ERα) was significantly up-regulated by 1.580 fold *(p=0.054).* In the group of proliferation and transcription factors, Insulin-Like Growth Factor 1 (IGF-1) was significantly down-regulated by 0.808 fold *(p=0.054),* the Serum Response Factor (SRF) was significantly up-regulated by 1.273 fold *(p=0.022)* and CCAAT/Enhancer Binding Protein D (CEBPD) was significantly down-regulated by 0.612 fold *(p=0.017).* The angiogenesis regulators FLT-1, Vascular Endothelial Growth Factor A (VEGF-A) and Endothelial Nitric Oxide Synthase 3 (eNOS3) were significantly down-regulated by 0.726 fold *(p=0.029),* 0.736 fold *(p=0.025)* and 0.632 fold *(p=0.024)* respectively. The Apolipoprotein A1 (APOA-1) which is involved in lipogenesis was significantly up-regulated by 2.041 fold *(p=0.040).*

Influence of treatment on gene expression in lung

Clenbuterol was originally designed for the treatment of asthma as bronchospasmolytic agent, highlighting the direct effect on lung tissue. However, there is not much known about the effect of steroid hormones on lung tissue. Therefore, the expression of the gene set that was chosen for clenbuterol was investigated in samples obtained from steroid-treated animals.

In the steroid-treated group, five genes were significantly up-regulated and two genes were significantly down-regulated. The Adrenergic Receptor β2 (ADRB2) was significantly down-regulated by 0.738 fold *(p=0.012).* In the group of proliferation and transcription factors, the Tumor Growth Factor β (TGF-β) and SMAD2 were significantly up-regulated by 1.459 fold *(p=0.039)* and 1.513 fold *(p=0.038)* respectively. Lipoprotein Lipase (LPL), a factor involved in lipogenesis was up-regulated by 1.603 fold *(p=0.007).* The tumor suppressor p53 was significantly up-regulated by 2.11 fold *(p=0.017).* Furthermore Glycerophosphodiester Phosphodiesterase Domain Containing 1 (GDPD1) was significantly down-regulated by 0.731 fold *(p=0.056)* and Serpin peptidase inhibitor, clade E (Serpin E1) was significantly up-regulated by 1.349 fold *(p=0.015)* respectively.

In the clenbuterol treated animals, three genes were significantly up-regulated and four genes were significantly down-regulated. GRα was significantly down regulated by 0.593 fold *(p=0.003).* The proliferation factor IGF-1 receptor (IGF-1R) was significantly down regulated by 0.643 fold *(p=0.011).* IRβ, a factor involved in glucose metabolism was significantly down-regulated by clenbuterol treatment by 0.742 fold *(p=0.009).* The immune factor Colony Stimulating Factor 2 (CSF2) was significantly up-regulated by 1.901 fold *(p=0.033).* The structure, protein Collagen 3A1 (Col3A1) was significantly up-regulated by 2.531 fold *(p=0.039).* Finally, Adenylate Kinase Isoenzyme 4, Mitochondrial (AK3L1) and Histamine Receptor H (HRH) were significantly up-regulated by 4.374 *(p=0.019)* and 2.531 *(p=0.039)* fold respectively.

A summary of regulated genes is given in Table 1.

Multivariate data analysis

To evaluate the potential of those gene expression results to develop a biomarker pattern, dynamic PCA and OPLS-DA were

applied. Using dynamic PCA, one set of biomarkers was identified in both tissues separately and in combination that resulted in the best separation of treated and untreated animals. In the shown graphs, blue dots represent control animals, red triangles steroid-treated animals and light green diamonds represent clenbuterol treated animals. In heart tissue, the combination of 22 genes (ANGPT1, bcl-xL, BMP-2, BMP-2R, CAST, c-Src, CYBB, EGF, ERα, ERβ, FAS, FLT1, IGF-1, IGF-2, IGFBP2, IRα, IRβ, p53, PI3K, PKB, SMAD 2, TIMP-2, VEGF 164) showed the best separation of untreated vs. steroid-treated animals. For the separation of control animals from clenbuterol treated individuals, a set of 12 genes, namely APOA1, CEBPD, eNOS, ERα, FAS, FLT-1, IGF-1, INHA, SRF, THBS, TIMP-2 and VEGFA has shown the best results. The corresponding dynamic PCAs are shown in Figure 1.

In lung tissue, a signature of 4 genes (FHIT, LPL, p53, PTGDS) could be identified for steroid treated animals and as signature of 8 genes (AK3L1, COL3A1, CSF2, GRα, HRH, IGF-1R, IRα, IRβ) for clenbuterol-treated animals. The resulting dynamic PCAs are shown in Figure 2.

In both tissues, a first biomarker pattern could be identified, but separation by PCA was not perfect meaning that control animals still group within the treated animals or vice versa. Therefore, qPCR data from lung and heart were combined in one dynamic PCA to evaluate, if a united biomarker signature can be identified. For the separation of control animals from steroid-treated animals, a signature of 17 genes, namely, ANGPT1, BMP2R, CAST, FLT-1, GRα, IRβ, RB1 and SMAD2 measured in heart tissue and ADRB2, FHIT, GDPD1, LPL, p53, PKC, SerpinE1, SMAD2 and TGFβ measured in lung tissue could be identified. For clenbuterol treatment, a signature of 9 genes could be identified, namely CEBPD, eNOS and SRF measured in heart tissue and AK3L1, CSF2, GRα, HRH, IGF-1R and IRβ measured in lung

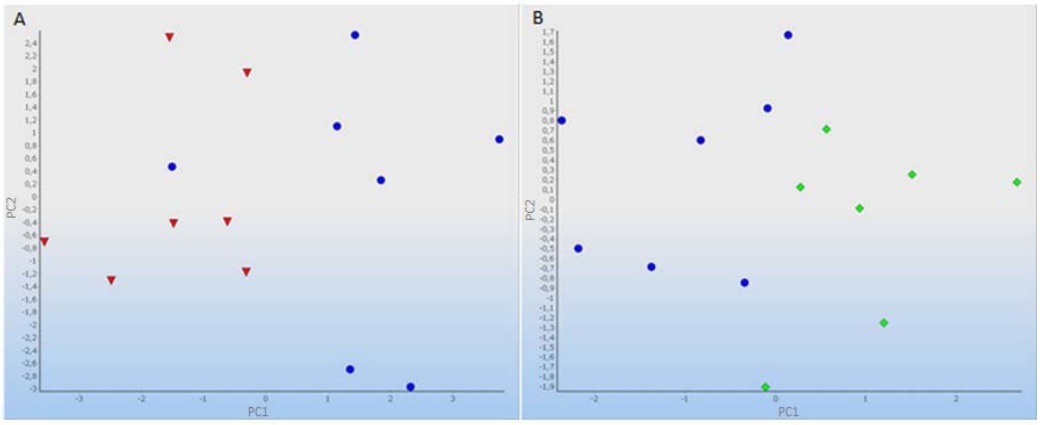

Figure 1: Dynamic principal component analysis of results obtained in heart tissue.
Figure 1A shows separation of the control group and the steroid treated group and
Figure 1B the separation of the control animals from the clenbuterol treated ones.
Animals of the control group are represented by blue dots, animals of the steroid-treated groups are represented by red triangles and clenbuterol treated animals are shown by light green diamonds.

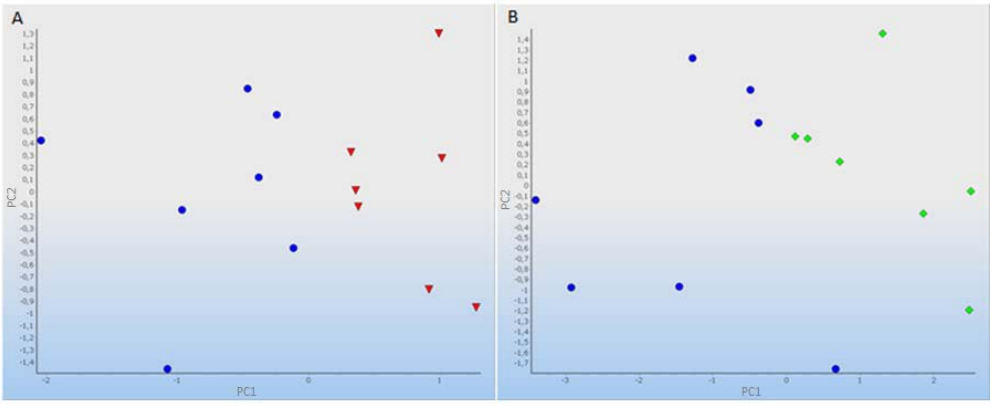

Figure 2: Dynamic principal component analysis of results obtained in lung tissue.
Figure 2A shows separation of the control group and the steroid treated group and
Figure 2B the separation of the control animals from the clenbuterol treated ones.
Animals of the control group are represented by blue dots, animals of the steroid-treated groups are represented by red triangles and clenbuterol treated animals are shown by light green diamonds.

Table 1: List of significantly regulated genes in heart and lung tissue.

Functional Group	Gene	Tissue	Treatment	Fold Regulation	*p-value*
Hormone Receptors	ADRB2	Lung	Steroid	0.738	*0.012*
	ERα	Heart	Clenbuterol	1.580	*0.054*
	GRα	Heart	Steroid	0.741	*0.021*
		Lung	Clenbuterol	0.593	*0.003*
Transcription Factors/ Proliferation	BMPR2	Heart	Steroid	0.792	*0.033*
	CEBPD	Heart	Clenbuterol	0.612	*0.017*
	IGF-1	Heart	Clenbuterol	0.808	*0.054*
	IGF-1R	Lung	Clenbuterol	0.643	*0.011*
	NFκB	Heart	Steroid	1.482	*0.031*
	RB1	Heart	Steroid	0.84	*0.034*
	SMAD2	Heart	Steroid	0.727	*0.016*
		Lung	Steroid	1.513	*0.038*
	SRF	Heart	Clenbuterol	1.273	*0.022*
	TGFβ	Lung	Steroid	1.459	*0.039*
Angiogenesis Regulators	ANGPT1	Heart	Steroid	0.606	*0.016*
	FLT1	Heart	Clenbuterol	0.726	*0.029*
			Steroid	0.695	*0.021*
	eNOS	Heart	Clenbuterol	0.632	*0.024*
	VEGFA	Heart	Clenbuterol	0.736	*0.025*
Immune Factors	CSF2	Lung	Clenbuterol	1.901	*0.033*
Oncogenes	p53	Lung	Steroid	2.110	*0.017*
Protein Metabolism/ Structural Proteins	CAST	Heart	Steroid	0.769	*0.033*
	COL3A1	Lung	Clenbuterol	1.810	*0.198*
Glucose Metabolism	IRβ	Heart	Steroid	0.777	*0.011*
		Lung	Clenbuterol	0.742	*0.009*
Lipid metabolism	APOA1	Heart	Clenbuterol	2.041	*0.04*
	LPL	Lung	Steroid	1.603	*0.007*
	PLCγ	Heart	Steroid	1.454	*0.059*
others	AK3L1	Lung	Clenbuterol	4.374	*0.019*
	ENO1	Heart	Clenbuterol	0.842	*0.063*
	GDPD1	Lung	Steroid	0.731	*0.056*
	HRH	Lung	Clenbuterol	0.636	*0.001*
	PKC	Heart	Steroid	1.399	*0.051*
	SerpinE2	Heart	Steroid	1.825	*0.015*
		Lung		1.349	*0.015*

tissue. The dynamic PCA for both treatments is shown in Figure 3. Additionally, a biomarker pattern of 23 genes (BMPR2, CAST, ENO1, NOS3, ERα, FAS, FLT-1, GRα, IGF-1, IRβ, PKB, RB-1, SMAD2 and TIMP-2 measured in heart tissue and ADRB2, Casp8, FHIT, GDPD1, GRα, IRα, PKC, PTGDS and SerpinE2 measured in lung tissue) that enabled separation of treated and untreated animals, independent of the applied substance, could be identified (Figure 3C).

To evaluate the predictive power of the biomarker signatures obtained by combining the results from both tissues, OPLS-DA analysis was performed. Regarding the resulting scatter plots, horizontal direction gives information about variations between the defined groups and vertical direction shows variations within the groups. Blue dots represent animals in the control group, red dots represent animals in the steroid group, light green dots represent clenbuterol-treated animals and dark green squares represent treated animals, independent of treatment. Figure 4 shows that, horizontal separation was achieved in both groups regarded separately (Figure 4A), whereas separation between control and the clenbuterol group was better (R2 (cum) = 0.929; Q2 (cum) = 0.824) than the separation of control group Vs the steroid group (R2 (cum) = 0.793; Q2 (cum) = 0.392) (Figure 4B). In the steroid group, one control animal, grouped with the treated animals. Regarding the variation within the groups, no difference could be observed. The predictive power of the third biomarker pattern (Figure 4C), enabling separation of treated from untreated animals independent of the applied substance (R2 (cum) = 0.830; Q2 (cum) = 0.520) was superior than for the steroid group regarded separately, but not as good as the pattern identified for clenbuterol alone.

Discussion

The misuse of growth-promoting substances in animal husbandry is an everlasting problem. Controls that are performed in routine monitoring are based on the detection of substance residues in different matrices, like urine, blood, hair, eyes or other tissues. But the identification of unknown xenobiotic drugs or hormone cocktails is still a problem [4]. An additional challenge is to distinguish between endogenous natural hormones and natural hormones that are applied exogenously. The idea of the identification of molecular biomarkers that are based on the investigation of physiological changes caused by the treatment with growth promoting agents has come into focus during the last years. Efforts in that field have already been made on the level of the metabolome [25], the proteome [7,8] and the transcriptome [3,9-11,19,26,27].

Within this study, new transcriptomic biomarker candidates for the detection of treatment with steroid hormones and clenbuterol in bovine heart and lung were quantified. Therefore, the targeted approach was applied, meaning that a list of potential biomarkers was selected by screening the literature for already published candidates or by choosing key factors from biochemical pathways that are known to be influenced by the treatments. The mRNA expression of those biomarker candidates was measured by RT-qPCR.

Regarding the physiological effects of treatment monitored by gene expression changes, it can be concluded that both treatments cause an increase in cell proliferation, an increased protein metabolism and an increase in the mobilization of lipid tissue. These effects go in line with the anabolic effect of the applied drugs.

To evaluate, if the quantified genes can be combined to a biomarker signature that enables the separation of treated from untreated animals, dynamic PCA was applied. In both tissues, a gene pattern could be specified for both treatments. All four biomarker patterns were not a simple combination of

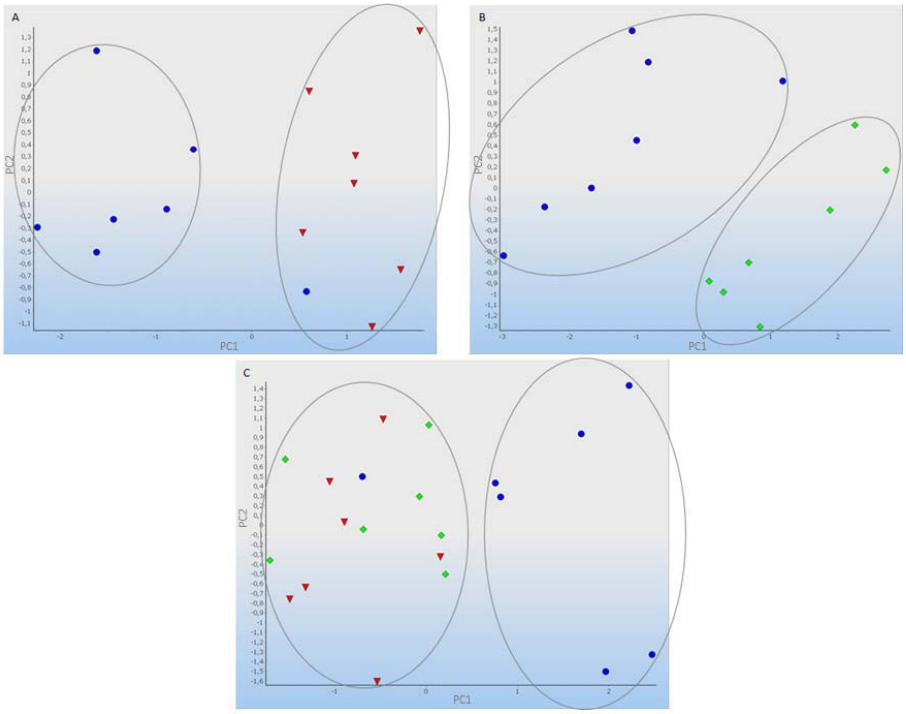

Figure 3: Dynamic principal component analysis of results obtained by the combination of qPCR data from heart plus lung tissue.
Figure 3A shows separation of control group and the steroid treated group,
Figure 3B the separation of the control animals from the clenbuterol treated ones and
Figure 3C the separation of control animals from treated individuals, independent of the applied substance.
Animals of the control group are represented by blue dots, animals of the steroid treated group are represented by red triangles and clenbuterol treated animals are shown by light green diamonds. Ellipses were drawn by hand to clarify a clear separation of treated and untreated animals.

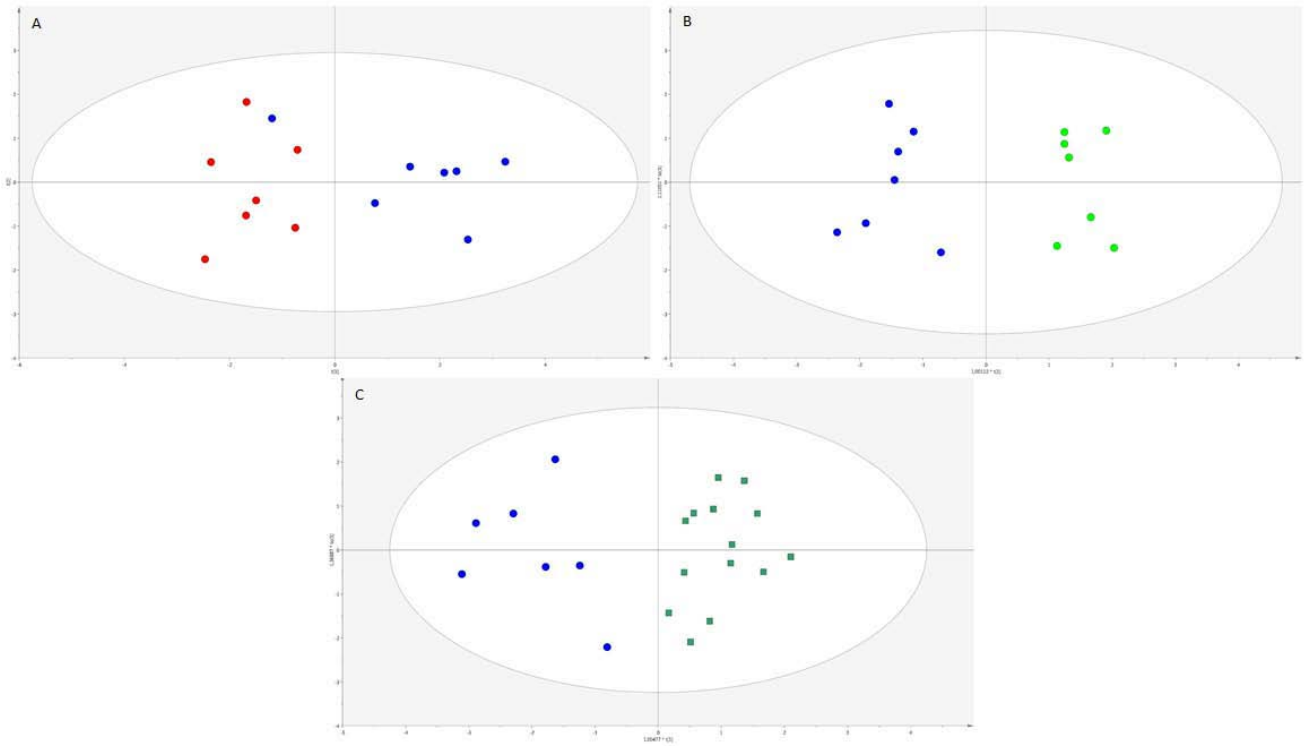

Figure 4: Dynamic principal component analysis of results obtained by the combination of qPCR data from heart plus lung tissue.

significantly regulated genes. Both gene patterns in lung tissue consisted of genes that were significantly regulated or regulated by trend *(p<0.1).* In heart tissue, also genes that showed a p-value higher than 0.1 were included in the pattern. Using the dynamic PCA algorithm, genes can either be excluded by a decreasing p-value or by an increasing distance of regulation [11]. Though, in both biomarker patterns identified in heart tissue, several genes were included, that has been chosen due to their distance of regulation. Significance was not reached for those genes due to inter-individual variability within the data set. The biomarker signatures identified in both tissues separately were not suitable to perfectly separate treated and untreated animals. To examine, if a better separation could be achieved by combining the results from both tissues, dynamic PCA was performed with qPCR data obtained in heart plus lung. Thereby a gene pattern could be identified to separate control animals from the steroid and clenbuterol-treated group. Additionally, a biomarker pattern that enabled separation of treated and untreated animals, independent of the applied substance was found. Identifying a substance independent biomarker pattern would be of advantage for establishing a first screening method in order to evaluate if an animal was treated with growth-promoting agents or not. Afterwards, an identification of the applied drug could be performed.

Predictive multivariate data analysis tools are helpful to determine the predictive power of a biomarker set. For that purpose, the use of OPLS-DA has already been shown to be adequate [21,22]. Regarding the resulting OPLS-DA analyses, best results were achieved for the combination of the results from heart and lung tissue for clenbuterol treatment. Good predictive power could also be confirmed for the biomarker signature that was independent of treatment. Only the results obtained for steroid treatment showed lower predictability. This may result from the control animal that clusters within the steroid treatment group.

These results show the suitability of primary target organs of the applied drugs for biomarker identification. Clenbuterol was designed to act as bronchodilator for the treatment of asthma. It directly acts via β2-adrenergic receptors in lung tissue resulting in relaxation of the bronchial muscles [17]. Another physiological effect of clenbuterol is an increased heart rate [28]. Both tissues are direct target organs of clenbuterol and were therefore regarded as ideal targets for biomarker identification. That assumption could be confirmed by the obtained results. Heart tissue as a potential source for gene expression biomarkers was chosen due to already published effects of steroid hormones on heart muscle [29]. During slaughter, increased heart sizes could also be observed (data not shown). As a consequence, major effects of steroid treatment were expected in heart tissue. Regarding lung tissue, effects of steroid hormones are rather unknown. However, a first biomarker pattern for the treatment with the steroid implant could be identified.

Conclusions

In this new study, gene expression biomarker candidates could be identified in heart and lung tissue to detect the illegal use of clenbuterol or steroids. The combination of the results of both tissues showed even better results than regarding each organ individually. Additionally, a biomarker pattern for the separation of untreated vs. treated animals independent of the applied substances could be identified. In liver tissue, similar results – a biomarker pattern independent of treatment - could be achieved, whereas in liver tissue, additional biomarkers could be identified using the non-targeted RNA-Sequencing approach [6]. The results of the current study showed that combining results from more tissues could also be a target-aimed.

To verify those biomarker signatures, more validation studies with other anabolic substances or application strategies like low dose cocktails will be necessary. Including gene expression data from other target organs, e.g. muscle tissue into the dynamic PCA analysis would also be a promising way to get a valid biomarker signature.

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References

1. Lange IG, Daxenberger A, Meyer HH. 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. 2001; 109(1): 53-65.
2. Moran C, Quirke JF, Prendiville DJ, Bourke S, Roche JF. The effect of estradiol, trenbolone acetate, or zeranol on growth rate, mammary development, carcass traits, and plasma estradiol concentrations of beef heifers. J Anim Sci. 1991; 69(11): 4249-58.
3. Riedmaier I, Tichopad A, Reiter M, Pfaffl MW, Meyer HH. Identification of potential gene expression biomarkers for the surveillance of anabolic agents in bovine blood cells. Anal Chim Acta. 2009; 638(1): 106-13. doi: 10.1016/j.aca.2009.02.014.
4. Cantiello M, Carletti M, Cannizzo FT, Nebbia C, Bellino C, Pié S, Oswald IP, et al. Effects of an illicit cocktail on serum immunoglobulins, lymphocyte proliferation and cytokine gene expression in the veal calf. Toxicology. 2007; 242(1-3): 39-51.
5. Riedmaier I, Becker C, Pfaffl MW, Meyer HH. The use of omic technologies for biomarker development to trace functions of anabolic agents. J Chromatogr A. 2009; 1216(46): 8192-9. doi: 10.1016/j.chroma.2009.01.094.
6. Pinel G, Weigel S, Antignac JP, Mooney MH, Elliott C, Nielen MWF, et al. Targeted and untargeted profiling of biological fluids to screen for anabolic practices in cattle. Trends in Analytical Chemistry. 2010; 29(11): 1269-80.
7. Ludwig SK, Smits NG, Cannizzo FT, Nielen MW. Potential of treatment-specific protein biomarker profiles for detection of hormone abuse in cattle. J Agric Food Chem. 2013; 61(19): 4514-9. doi: 10.1021/jf4004972.

8. Ludwig SK, Smits NG, van der Veer G, Bremer MG, Nielen MW. Multiple protein biomarker assessment for recombinant bovine somatotropin (rbST) abuse in cattle. PLoS One. 2012; 7(12): e52917. doi: 10.1371/journal.pone.0052917.

9. Becker C, Riedmaier I, Reiter M, Tichopad A, Groot MJ, Stolker AA, et al. Influence of anabolic combinations of an androgen plus an estrogen on biochemical pathways in bovine uterine endometrium and ovary. J Steroid Biochem Mol Biol. 2011; 125(3-5): 192-201. doi: 10.1016/j.jsbmb.2011.01.007.

10. Becker C, Riedmaier I, Reiter M, Tichopad A, Pfaffl MW, Meyer HH. Effect of trenbolone acetate plus estradiol on transcriptional regulation of metabolism pathways in bovine liver. Horm Mol Biol Clin Investig. 2010; 2(2): 257-65. doi: 10.1515/HMBCI.2010.029.

11. Riedmaier I, Spornraft M, Pfaffl MW. Identification of a potential gene expression biomarker signature in bovine liver to detect the abuse of growth promoters. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2014; 31(4): 641-9. doi: 10.1080/19440049.2014.886341.

12. Wallukat G. The beta-adrenergic receptors. Herz. 2002; 27(7): 683-90.

13. Mersmann HJ. Overview of the effects of beta-adrenergic receptor agonists on animal growth including mechanisms of action. J Anim Sci. 1998; 76(1): 160-72.

14. Reeds PJ, Hay SM, Dorward PM, Palmer RM. The effect of beta-agonists and antagonists on muscle growth and body composition of young rats (Rattus sp.). Comp Biochem Physiol C. 1988; 89(2): 337-41.

15. Sillence MN, Matthews ML, Spiers WG, Pegg GG, Lindsay DB. Effects of clenbuterol, ICI118551 and sotalol on the growth of cardiac and skeletal muscle and on beta 2-adrenoceptor density in female rats. Naunyn Schmiedebergs Arch Pharmacol. 1991; 344(4): 449-53.

16. Nieminen MS, Rämö MP, Viitasalo M, Heikkilä P, Karjalainen J, Mäntysaari M, et al. Serious cardiovascular side effects of large doses of anabolic steroids in weight lifters. Eur Heart J. 1996; 17(10): 1576-83.

17. Roets E, Burvenich C. Effect of clenbuterol on beta-adrenoreceptors and adenylate cyclase activity in smooth muscle and epithelium of the trachea of calves. Vet Q. 1993; 15(4): 180-1.

18. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009; 55(4): 611-22. doi: 10.1373/clinchem.2008.112797.

19. Riedmaier I, Benes V, Blake J, Bretschneider N, Zinser C, Becker C, et al. RNA-sequencing as useful screening tool in the combat against the misuse of anabolic agents. Anal Chem. 2012; 84(15): 6863-8. doi: 10.1021/ac301433d.

20. Riedmaier I, Pfaffl MW. Transcriptional biomarkers--high throughput screening, quantitative verification, and bioinformatical validation methods. Methods. 2013; 59(1): 3-9. doi: 10.1016/j.ymeth.2012.08.012.

21. Regal P, Anizan S, Antignac JP, Le Bizec B, Cepeda A, Fente C. Metabolomic approach based on liquid chromatography coupled to high resolution mass spectrometry to screen for the illegal use of estradiol and progesterone in cattle. Anal Chim Acta. 2011; 700(1-2): 16-25. doi: 10.1016/j.aca.2011.01.005.

22. Blokland MH, Van Tricht EF, Van Rossum HJ, Sterk SS, Nielen MW. Endogenous steroid profiling by gas chromatography-tandem mass spectrometry and multivariate statistics for the detection of natural hormone abuse in cattle. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2012; 29(7): 1030-45. doi: 10.1080/19440049.2012.675593.

23. Bylesjo M, Rantalainen M, Cloarec O, Nicholson JK, Holmes E, Trygg J. OPLS discriminant analysis: combining the strength of PLS-DA and SIMCA classification. Journal of Chemometrics. 2006; 20(8-10): 341-51.

24. Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med. 2006; 27(2-3): 126-39.

25. Dervilly-Pinel G, Courant F, Chéreau S, Royer AL, Boyard-Kieken F, Antignac JP, et al. Metabolomics in food analysis: application to the control of forbidden substances. Drug Test Anal. 2012; 4 Suppl 1: 59-69. doi: 10.1002/dta.1349.

26. Becker C, Riedmaier I, Reiter M, Tichopad A, Pfaffl MW, Meyer HH. Changes in the miRNA profile under the influence of anabolic steroids in bovine liver. Analyst. 2011; 136(6): 1204-9. doi: 10.1039/c0an00703j.

27. Riedmaier I, Reiter M, Tichopad A, Pfaffl MW, Meyer HH. The potential of bovine vaginal smear for biomarker development to trace the misuse of anabolic agents. Exp Clin Endocrinol Diabetes. 2011; 119(2): 86-94. doi: 10.1055/s-0030-1267239.

28. Meyer HH. Biochemistry and physiology of anabolic hormones used for improvement of meat production. APMIS. 2001; 109(1): 1-8.

29. Payne JR, Kotwinski PJ, Montgomery HE. Cardiac effects of anabolic steroids. Heart. 2004; 90(5): 473-5.