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Impact of anabolics on miRNA and mRNA abundance in steroid target organs

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Abbreviations

ACTB	Actinβ
Alk	anaplastic lymphoma receptor tyrosine kinase
APO	apolipoprotein
bcl-2	B-cell CLL/lymphoma 2
BMP	bone morphogenetic protein
bp	base pair
c-fos	c-fos transcription factor
c-kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogen homolog
CAST	calpastatin
CL	corpus luteum
COX	cyclooxygenase
CTSB	cathepsin B
CV	coefficient of variation
CYP	cytochrom P450
DMSO	dimethylsulfoxid
(c)DNA	(complementary) desoxyribonucleic acid
ds	double stranded
EIA	enzyme immunoassay
EU	European Union
Ago	argonaute protein
ANGPT	angiopoietin
AP-1	activator protein 1
AR	androgen receptor
bcl-xl	B-cell leukemia/lymphoma x
BMPR	bone morphogenetic protein receptor
c-jun	c-jun transcription factor
c-myc	myelocytomatosis cellular oncogen homolog
CASP	caspase
CEBP	CCAAT/enhancer binding protein
Cq	quantitative cycle
CTSL	cathepsin L
CYL	cyclin
DEGMBE	diethylen glycol monobuthylether
dNTP	desoxyribonucleosid-triphosphat
E2	estradiol-17β
ER	estrogen receptor

FAS	TNF receptor superfamily member 6	FASL	TNF receptor superfamily member 6 ligand
FDFT	farnesyldiphosphat-farnesyltransferase	FGF	fibroblast growth factor
FLT-1	fms-related tyrosine kinase receptor	FLK-1	kinase insert domain
GDF	growth differentiation factor	FSHR	follicle stimulating hormone receptor
GR	glucocorticoid receptor	GHR	growth hormone receptor
HCA	hierarchical cluster analysis	H3	histon 3
HGF	hepatocyte growth factor	HCC	hepatocellular carcinoma
HMGCoA-R	3-hydroxy-methylglutaryl coenzym-A-reductase	HK	hexokinase
HRE	hormone responsive elements	HMGCoA-S	3-hydroxy-methylglutaryl coenzym-A-synthase
HSD17B	17β-hydroxy steroid dehydrogenase	HSD3B	3β-hydroxy steroid dehydrogenase
IGF-BP	insulin-like growth factor binding protein	HSP	heat shock protein
IL	interleukin	IGF	insulin-like growth factor
IR	insulin receptor	IGF-1 R	insulin-like growth factor 1 receptor
LDH	lactatdehydrogenase	INHA	inhibin A
LIPC	hepatic lipase	kb	kilobases
LPL	lipoprotein lipase	LHR	luteinizing hormone receptor
MMP	matrix metalloproteinase	LTF	lactotransferrin
miRNA	microRNA	mRNA	messenger ribonucleic acid
NF-κB	nuclear factor κB	M-MLV H^-	moloney murine leukemia virus RNase minus
nm	nano meter	nt	nucleotide

OD	optical density	p53	p53 tumor suppressor gene
PCA	principal components analysis	PHB	prohibitin
		PR	progestin receptor
qPCR	quantitative polymerase chain reaction	RB-1	retinoblastoma 1
		RBBP	retinoblastoma binding protein
RG	reference gene		
rRNA	ribosomal RNA	RIN	RNA integrity number
RQI	RNA quality index	RISC	RNA induced silencing complex
RT	reverse transcription		
S5A1	5α-reductase	SCAP	SREBP cleavage activating protein
SHBG	steroid hormone binding globulin		
		SP-1	specificity protein 1
SREBP	sterol regulatory element binding protein	STAR	steroidogenic acute regulatory protein
T_M	annealing temperature	TAT	tyrosine aminotransferase
TBA	trenbolone acetate	TGF	transforming growth factor
THBS	thrombospondin	TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumor necrosis factor		
UBQ	ubiquitin	UTR	untranslated region
UV	ultra violett	v-myb	myeloblastosis viral oncogen homolog
VEGF	vascular endothelial growth factor		
		WBC	white blood cells

Zusammenfassung

Steroidale Sexualhormone sind evolutionär tief verwurzelte Moleküle und bei allen Wirbeltieren zu finden. Neben ihren wichtigen Funktionen in der männlichen und weiblichen Reproduktion zeigen v.a. Androgene und Estrogene anabole Eigenschaften durch vermehrte Proteinretention und Mobilisierung von Fettdepots. Deswegen sind natürliche und xenobiotische Steroidhormone im Sport („Doping“) und der Tiermast zur Leistungs- und Wachstumsförderung gewinnbringend einsetzbar. Zur Gewährleistung der Fairness im Wettkampf ist Doping im Leistungssport nicht zulässig. Ebenso ist die Anwendung von wachstumsfördernden Substanzen in der Tiermast EU-weit seit 1988 verboten, da die Gesundheitsrisiken für den Verbraucher durch Hormonrückstände im Fleisch kaum abgeschätzt werden können. Die Einhaltung dieser Richtlinien wird sowohl im Sport als auch in der kommerziellen Landwirtschaft hauptsächlich durch chromatographische Techniken in Kombination mit Massenspektrometrie [z.B. (HP)LC-MS-MS] kontrolliert, mit denen Rückstände der verwendeten Substanz detektiert werden. Solche Analyseverfahren können durch das Aufkommen neuer Substanzen sowie die Anwendung von Hormoncocktails umgangen werden. Deswegen besteht die Notwendigkeit, innovative und präzise Nachweismethoden für ein engmaschiges Kontrollsystem zu etablieren. Eine Möglichkeit zum Nachweis einer illegalen Steroidbehandlung wäre die Einbeziehung physiologischer Veränderungen, die anabole Substanzen im Organismus hervorrufen. Einige Studien gaben erste Hinweise, dass die Messung von Veränderungen des Transkriptoms („transcriptomics“) durch quantitative RT-PCR (RT-qPCR) hierbei einen vielversprechenden Ansatz darstellt, jedoch ist wenig über die Auswirkungen von anabolen Steroiden auf die Genexpression im Rind bekannt.

In der aktuellen Arbeit wurde untersucht, ob es möglich ist, in den gewählten Zielorganen (Leber, weiblicher Geschlechtstrakt) ein gewebespezifisches Genexpressionsmuster zu ermitteln, das einen möglichen Biomarker zum Nachweis der exogenen Anwendung von anabol wirksamen Substanzen darstellt. Biostatistische Methoden zur Erkennung von Expressionsmustern, wie z. B die Hauptkomponentenanalyse (PCA), wurden eingesetzt, um die gewünschten Informationen aus der Vielzahl der gemessenen Gene zu extrahieren.

Zum ersten Mal wurde auch die Expression von miRNAs unter dem Einfluss anaboler Steroide in der bovinen Leber gemessen. Diese RNA-Spezies erwies sich in den letzten 10 Jahren durch ihre Gewebe- und Krankheitsspezifität als vielversprechende Biomarker in der klinischen Diagnostik. Möglicherweise zeigen diese Moleküle auch erhebliches Potential zur Nutzung als Biomarker in der Überwachung der illegalen Anabolikaanwendung.

Der Einfluss der Qualität des Probenmaterials auf die Genexpressionsanalyse wurde in vorherigen Studien nur für die Messung der mRNA erörtert. Wenig ist hingegen zur Anfälligkeit von kleinen RNAs gegenüber einer Degradierung bekannt. In dieser Arbeit wurde das Ausmaß dieses Einflusses auf die Bestimmung der Konzentration sowie die Amplifizierung von miRNAs mittels qPCR untersucht, um in späteren physiologischen Untersuchungen valide Expressionsergebnisse zu erhalten.

Es konnte gezeigt werden, dass die Degradierung des Probenmaterials ähnlich wie bei der mRNA Analytik einen immensen Einfluss auf die Konzentrationsmessung sowie die Amplifizierung der miRNA hat. Es ist somit bei Untersuchung der miRNA ebenso wichtig, eine hohe Qualität des Probenmaterials zu erhalten und RNA Integritätsmessungen durchzuführen.

Die Tierversuche an post-pubertären Färsen ergaben erhebliche Veränderungen der Genexpression als Folge der Anwendung anaboler Steroide. Hierbei konnten die physiologischen Aussagen der mRNA Expressionsanalyse durch die Messung des miRNA Profils bestätigt werden, wodurch dargelegt wurde, dass sich auch die miRNA Expression als Indikator für die Anwendung von anabolen Steroiden nutzen lassen könnte. Durch die Auswertung der Expressionsdaten mittels PCA konnten behandelte Tiere erfolgreich von Kontrolltieren getrennt werden. Hierbei zeigte sich vor allem die Kombination von Ergebnissen der mRNA und miRNA Expression desselben Transkriptoms als vielversprechende Möglichkeit. Diese Ergebnisse deuten darauf hin, dass v.a. in Leber und Ovar möglicherweise ein Genexpressionsmuster (mRNA und miRNA) gefunden werden kann, das als Biomarker zur Aufdeckung der illegalen Steroidanwendung dienen könnte.

Die Untersuchung des Ovars pre-pubertärer Kälber zeigte, dass entweder diese Altersklasse oder der unreife Reproduktionstrakt weniger geeignet sind, den Nachweis einer anabolen Behandlung in einem niedrig dosierten Bereich durch Transkriptomanalysen zu erbringen.

Abstract

Steroidal sex hormones are deep-rooted in the evolution and can be found in all vertebrates. Besides their important function in male and female reproduction, especially androgens and estrogens exhibit anabolic characteristics by increasing protein retention and mobilisation of fat stores. Due to these properties, natural and xenobiotic steroid hormones can be used profitably in human sports ("doping") as well as in animal husbandry to increase growth rate and performance. To obtain fairness in competition, doping is not permitted in competitive sports. Furtheron, in the EU the application of growth promoting agents is forbidden in animal husbandry since 1988, as the health risk for the consumer due to hormone residues in edible tissue can hardly be estimated. The compliance with these guidelines is mainly controlled in sports as well as in commerical agriculture by chromatographical methods in combination with tandem mass spectrometry [e.g. (HP)LC-MS-MS] by directly detecting the applied substance. These techniques can be eluded by the emerging of new substances or the application of hormone cocktails. Hence, there is a need for establishing innovative and precise detection methods for a small meshed control system. One possibility would be to detect misuse by including the physiological changes in the organism triggered by exogenous steroid hormones. Some studies showed that investigating differences in the transcriptome ("transcriptomics") using quantitative RT-PCR (RT-qPCR) might be a promising approach. However, few is known about gene expression changes due to anabolic steroids in cattle.

In the actual thesis, it was investigated, if it would be possible to find tissue specific gene expression patterns in the target organs (liver, female reproductive tract), which might be used as biomarker for anabolic treatment screening. Appropriate bio-statistical tools should be employed to gain the designated information from gene expression data.

For the first time, miRNA expression patterns have been measured under the influence of anabolic steroids in bovine liver. This RNA-species has been shown to be auspicious as biomarker in clinical diagnostics due to its tissue und disease specificity. Possibly, these molecules have plenty of potential to be used as biomarkers in surveillance of anabolic misuse.

The influence of sample quality on gene expression analysis has been described in previous studies only for mRNA measurement. However, few is known about the susceptibility of miRNAs to degradation. The degree of this influence on concentration measurement and amplification of miRNAs using qPCR was investigated prior to physiologically based miRNA expression measurement to gain valid results.

It could be shown that degradation has a distinct influence on determination of miRNA concentration and miRNA amplification similar to mRNA. So, sustaining of high-quality sample material and RNA integrity measurement is also important in miRNA studies.
Animal studies on post-pubertal heifers revealed significant changes in gene expression due to anabolic action on the gene expression level in liver and reproductive organs. Herein, the physiological discussions drawn for mRNA could be supported by changes in the miRNA profile in liver of Nguni heifers showing distincly that also miRNA expression could be a suitable indicator for transcriptional changes due to anabolic steroid hormone action.
It could be demonstrated that evaluation of gene expression data using PCA could successfully be used to separate treated animals from control animals. Furthermore, the combination of mRNA and miRNA expression results from the same transcriptome seems to be the most promising approach. These results indicate that it might be possible to find a gene expression pattern, especially in liver and ovary (mRNA and miRNA), which might be used for anabolic treatment screening.
Studies on ovary of pre-pubertal calves illustrated that either this age class or the immature reproductive tissue might be less suitable for the detection of anabolic misuse in the low-range level using transcriptomics.

1 Introduction

1.1 Anabolic steroids and their use in animal husbandry

In the organism, steroid hormones are produced from the precursor cholesterol and can be divided into five groups, which are physiologically relevant: mineralocorticoids, glucocorticoids, androgens, estrogens and gestagens [1]. Steroidal reproductive hormones (androgens, estrogens, gestagens) are deep-rooted in evolution and can be found in all marine and terrestrial vertebrates playing a major role in the regulation of sexual diversity including metabolism, behaviour and morphology [2; 3]. Androgens and estrogens exhibit important functions in both males and females, but estrogens prevail in females and androgens are predominantly expressed in male individuals. In females, estradiol-17β (E2) (figure 1A) prepares the organism for successful reproduction in combination with progesterone by regulation of the estrus cycle. During pregnancy, E2 initiates protein and mineral retention for the development of the embryo and to prepare the upcoming lactation peroid. Testosterone (figure 1B), which is the main reproductive hormone in males, is responsible for spermatogenesis.

Figure 1: Molecular structures of estradiol-17β (A) and testosterone (B) [1]

Besides their important role in reproduction, androgens and estrogens exhibit anabolic effects on various tissues. Herein, these molecules increase growth rate by enhancing protein and amino acid retention and initiate the mobilisation of fat stores. Due to these abilities, natural and xenobiotic [e.g. trenbolone acetate (TBA)] steroid hormones can be used in different fields. In human sports, the application of anabolic steroids is called doping and was exerted in many disciplines (athletics, cycling, bodybuilding, etc.). To obtain fairness in competition, doping is not permitted in competitive sports. Anabolic agents are also used as growth promoters in animal husbandry due to their capacity to increase growth rate, the amount of lean body mass, and feed efficiency [1; 4]. As fewest steroid hormones are orally active, in the majority of cases, growth promoters are applied via implantation or injection.

According to the profitability in lean meat production, the application of these drugs is admitted and widely spread in countries like the USA, Canada, South Africa or Mexico. In the European Union (EU), the use of anabolic agents is only licensed for zootechnical purposes like estrus synchronisation and estrus induction, but forbidden in food production since 1988, because the potential health risks for the consumer due to hormone residues in edible tissues can hardly be estimated.

1.2 Transcriptomics as a potential method to detect biomarkers for anabolic treatment

The misuse of anabolic steroids is a potential problem and the establishment of innovative and sensitive detection methods is needed. In human sports as well as in commercial animal husbandry, the misuse of anabolics or other growth promoting agents is currently controlled in body fluids (urine, plasma) by chromatographic techniques in combination with (tandem) mass spectrometry e.g. (HP)LC-MS-MS [5-8]. These methods are able to directly detect the applied substances and thereby to control the compliance with certain thresholds. Unfortunately, this can be eluded by the emergence of new, synthetic substances with unknown molecular properties (“designer drugs”) or the application of hormone cocktails as steroid hormones act synergistically and the concentration of each component of the cocktail might be under the defined threshold [9]. Hence, there is a need for the establishment of more sensitive detection methods. One possibility would be to detect anabolic action by including the physiological effects on the organism triggered by exogenously applied steroid hormones. Various groups are doing research on this approach by monitoring regulatory physiological processes like transcription, translation/protein synthesis and metabolism. For analysis, different -omic technologies are available including transcriptomics, proteomics and metabolomics [10-14]. Using transcriptomics might be a promising possibility to detect anabolic treatment, as it is known that steroid hormones act as transcription factors and thereby directly influence the expression of specific genes. The majority of steroid hormone receptors is found in the cytoplasm entering the nucleus after activation, but estrogen receptors (ER) could also directly be present in the nucleus [15]. Due to their lipophilic properties, steroid hormones enter the cell and/or the nucleus by passive diffusion through the cellular membrane.

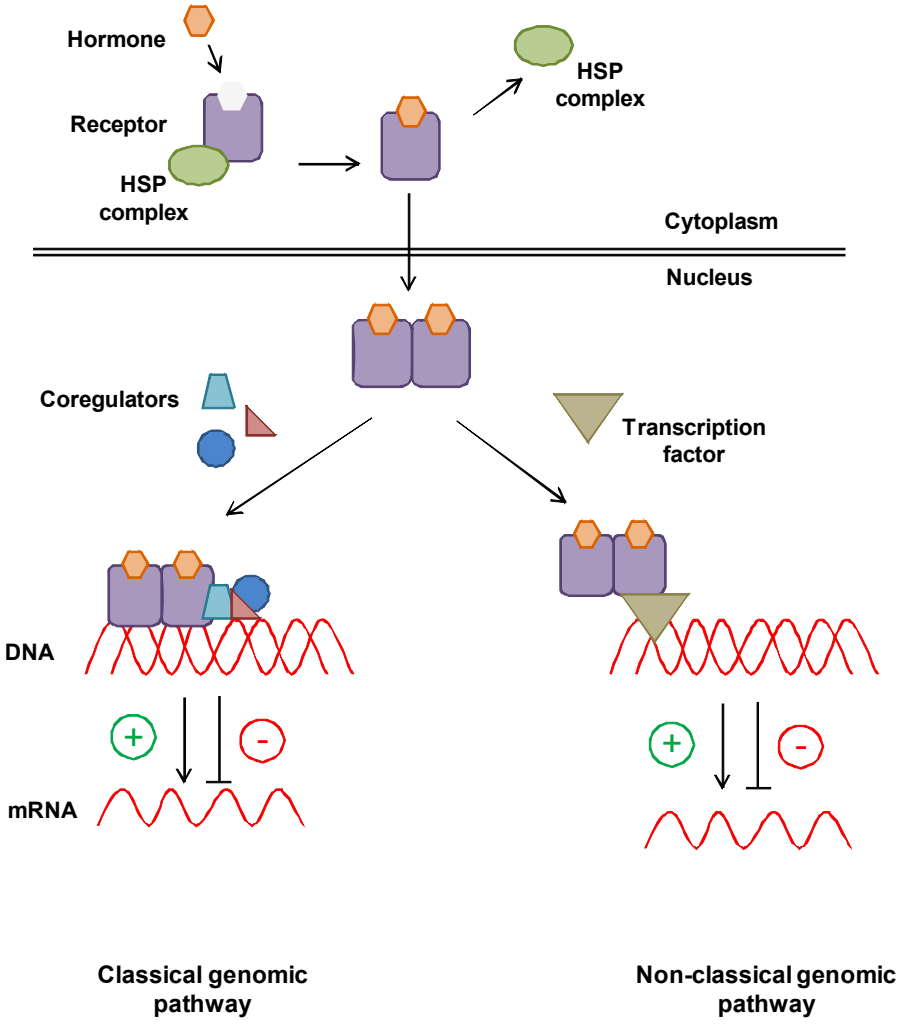

Figure 2: Mechanism of steroid hormone binding to a cytoplasmatic receptor and following classical and non-classical regulation of transcription

Prior to hormone binding, steroid hormone receptors are complexed with different heat shock proteins (HSP90, HSP56, HSP70). The HSP complex is disunited due to conformational changes after binding of the hormone to its respective receptor. The steroid hormone-receptor complex is translocated to the nucleus and forms homodimers. In the nucleus, steroid hormone-receptor complexes can exert their effect on gene expression in two different ways (figure 2). The classical genomic pathway is well characterized. Herein, the receptor dimers bind to specific hormone responsive elements (HRE) in the promoter region of their target gene via zinc finger motifs in their DNA binding domain. Following DNA binding, different coregulators are recruited, which either activate or repress expression of the target gene [10].

In the non-classical genomic pathway, steroid hormones regulate transcription by induction of other nuclear transcription factors like Sp-1, Ap-1 or NF-κB [16-18]. In turn, these regulate the transcription of their respective target genes by blocking or induction of the assembly of the RNA polymerase II [19].

Measurement of gene expression data in blood has been shown to be an auspicious approach to discover illegal application of growth promoters. However, blood is not directly dependent on steroid hormone action, but rather a transport organ for synthesized mRNAs. So, more distinct changes may be detectable in primary or secondary hormone-dependent tissues like reproductive organs and liver. Liver plays an important role in the metabolism of steroid hormones, e.g. hormone decomposition, and production of cholesterol as precursor of all steroids. Additionally, many metabolic pathways in liver are controlled by steroid hormone action. Reproductive organs are primary hormone-dependent tissues and are thought to be the most sensitive organs due to the high steroid hormone receptor expression [20]. Additionally, former investigations showed the highest differences in gene expression levels under the influence of anabolic steroids in tissues from the reproductive tract [21].

After gaining gene expression data, suitable bio-statistical methods are needed to extract the required information from the results. Typically for biomarker research, not only one factor, but a pattern with multiple factors influenced by the specific treatment is established. Different methods for pattern recognition like principal components analysis (PCA) or hierarchical cluster analysis (HCA) can be used. These techniques allow to reduce the multidimensionality of expression data from a multitude of genes to a two dimensional plot [22]. PCA has already been effectively used for biomarker research concerning the application of anabolic steroids [23].

1.3 miRNAs as a promising new target in gene expression analysis for biomarker detection

MicroRNAs (miRNAs) are small RNAs with a length of approximately 22 nucleotides (nt), those are thought to be involved in the regulation of many important physiological processes like growth and development. 1-3% of the human genome are estimated to encode for miRNAs and about 30% of the genes are predicted to be regulated by miRNAs [24].

These molecules were already described in 1993 [25], the name “miRNAs” was primary alluded in 2001, and the analytical interest in valid miRNAs quantification arose over the past years.

miRNA biogenesis

The information for miRNA often lies in intronic regions of protein-coding genes (~90%), but could also derive from independent transcription sites lying quite distant from assigned genes. Thereby in the transcriptional process (figure 3), the primary miRNA hairpin transcripts (pri-miRNAs) could either share their promoter region with their pre-mRNA host genes or are transcribed from their own promoters. The transcription of most miRNAs is mediated by RNA polymerase II, a minor group can also be transcribed by RNA polymerase III.

The pri-miRNAs with a length of usually several kilobases (kb) are further processed by Drosha, an RNase III endonuclease. Drosha cuts the pri-miRNAs at the stem of the hairpin, generating a small stem-loop structure, the 60-70 nt miRNA precursors (pre-miRNAs), with a 5´phosphate and an ~ 2nt 3´overhang at the base of the stem-loop, which is typical for RNase III nuclease cleavage. The pre-miRNA, which represents an imperfect hairpin structure, is actively exported from the nucleus to the cytoplasm by the transport protein Exportin-5. In the cytoplasm, the enzyme Dicer, which is also an RNase III endonuclease, cuts the loop from the stem-loop structure producing the double-stranded miRNA:miRNA*-duplex with an 5´phosphate and an ~ 2 nt 3´overhang on both strands. After unwinding of the duplex structure by a helicase, the mature miRNA is incorporated in the RNA-induced silencing complex (RISC). RISC comprises of Argonaute proteins (Ago), an endonuclease and other non-specific protein, whose function is not yet determined. The RISC assembly of one strand of the double-stranded duplex is based on the thermodynamic features of the duplex. Following the general rule, that strand is estimated as the mature miRNA and is incorporated, which is less tightly paired on its 5´end. The complementary miRNA* is thought to be degraded, but its fate is not fully clarified and it is not clear, if this strand has other functional properties [24; 26; 27].

Only the seed sequence of the mature miRNA (6-8 nt at the 5´end) binds perfectly to the 3´untranslated region (UTR) of the target mRNA [28]. The regulatory action is dependent on the complementarity of the remaining miRNA sequence to the target mRNA. Herein, miRNAs can regulate gene expression in two different ways. If the miRNA is perfectly or nearly perfectly complementary to its correspondent target, the mRNA will be cleaved by the nuclease activity of RISC. This effect is typically for the action of miRNAs in plants and lower organisms. In higher vertebrates, regulation of gene expression by miRNAs is mostly caused by translational repression after incomplementary binding of the mature miRNA incorporated in RISC to its target mRNA, as it is depicted in figure 3.

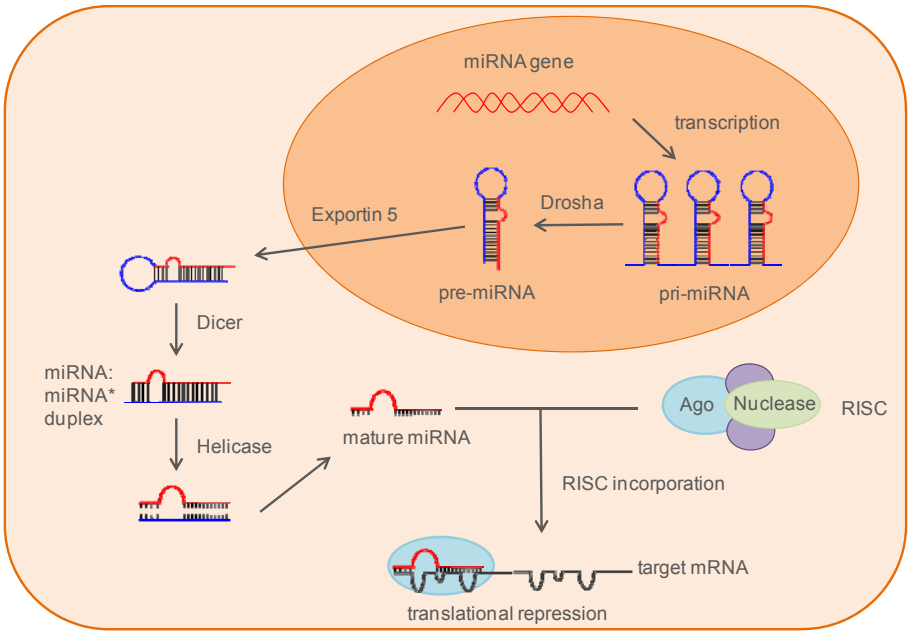

Figure 3: miRNA biogenesis and the process of translational repression by the mature miRNA

miRNAs as biomarkers in diagnostics

The interest in valid miRNA analysis rose over the past ten years especially in the medical diagnostic fields. Concerning functional studies, particularly the investigation of miRNA expression profiles is of great interest, because miRNAs are involved in the genesis of several pathological events in humans. Besides other diseases (e.g. diabetes), miRNAs are implicated in the pathogenesis of different cancer types (lung, breast, prostate, gastrointestinal tract) and therefore could be used as clinical markers in diagnostic [29-31]. Screening of miRNA expression gives exceptional insights in differentiation stages and developmental lineage of tumors [27].

In cancer diagnosis, miRNA profiles have proven their superiority over mRNA profiles as miRNA patterns seem to be generated in a tissue- and disease-specific manner, which is not the case for other established biomarkers [27; 28]. For example, Lu et al. were able to discriminate gastrointestinal cancer tissue from non-gastrointestinal cancer tissue by their specific miRNA profile [30]. This was not possible when screening the same samples with a multitude of 16000 mRNAs. Furthermore, after the transcriptional processing (pri-miRNA, pre-miRNA), there are no further modifications in the mature miRNAs and thereby no different variants e.g. splice variants of the same molecule facilitating an accurate detection [32].

Especially the discovery of circulating miRNAs in plasma was promising for early diagnosis as this would offer a minimal invasive possibility for early cancer detection. Several groups

showed that it would be possible to identify prostate cancer patients by measuring the plasma levels of certain miRNAs [31; 33].
The idea of establishing biomarkers is also present in veterinary drug analysis, for example in surveillance of misuse of illegal growth promoters like anabolic steroids. The potential of transcriptomics as a method to evaluate biomarkers for anabolic treatment has been shown in several studies [21; 23]. So, the investigation of miRNA profiles might also a new and precise method to trace anabolic treatment.

1.4 Importance of RNA quality control in gene expression analysis

The importance of high quality sample material, i.e. non-degraded or fragmented RNA, in transcriptomics is well documented. Various studies showed a distinct influence of total RNA integrity on the performance of classical gene expression profiling [34; 35]. Hence, the analysis of RNA quality is a valuable tool in the preparation of methods like RT-qPCR and microarray analysis. RNAs are very sensitive molecules and the ubiquitous occurrence of nucleases poses a constant risk of RNA degradation. For this reason, cautious handling in every single pre-PCR step (e.g. sampling, storage and extraction) is important as only experiments conducted with high quality starting material provide reliable results. The recently published guidelines for “minimum information for publication of quantitative real-time PCR experiments” (MIQE guidelines) demand a higher transparency of the pre-PCR steps like the documentation of sample quality [36; 37]. These guidelines are supposed to give recommendations for authors, which details are necessary to be declared in a scientific publication. This should guarantee to get a standardized paperwork for gene expression experiments to help the reader to evaluate and reproduce published results, to promote consistency between laboratories, and to increase experimental transparency.
The term RNA quality is defined as the composition of RNA purity and RNA integrity. RNA purity can be measured photometrically. Herein, the optical density (OD) is measured at different wave lengths: 230 nm (absorption of contaminants & background absorption), 260 nm (absorption maxima of nucleic acids), 280 nm (absorption maxima of proteins), and 320 nm (absorption of contaminants & background absorption). The $OD_{260/280}$ ratio is used as indicator for RNA purity. A ratio higher than 1.8 is assumed as suitable for gene expression measurements [38; 39]. The $OD_{260/230}$ and the $OD_{260/320}$ should be maximized as these represent the degree of background absorption and contaminants.
For standardized RNA integrity control, lab-on-chip technology for automated capillary electrophoresis is state of the art and is recommended. Different lab-on-chip instruments are

commercially available e.g. the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and the Experion (Bio-Rad Laboratories, Munich, Germany). Both devices are sensitive, highly reproducible and suitable for a reliable quality control of RNAs [114]. For visualization and better interpretation, an electropherogram (figure 4A) and a virtual gel-like image (figure 4B) are generated. The 28S/18S ribosomal RNA (rRNA) ratio is calculated by assessing the peaks recorded in the electropherogram and the bands occurring on the gel-like image.

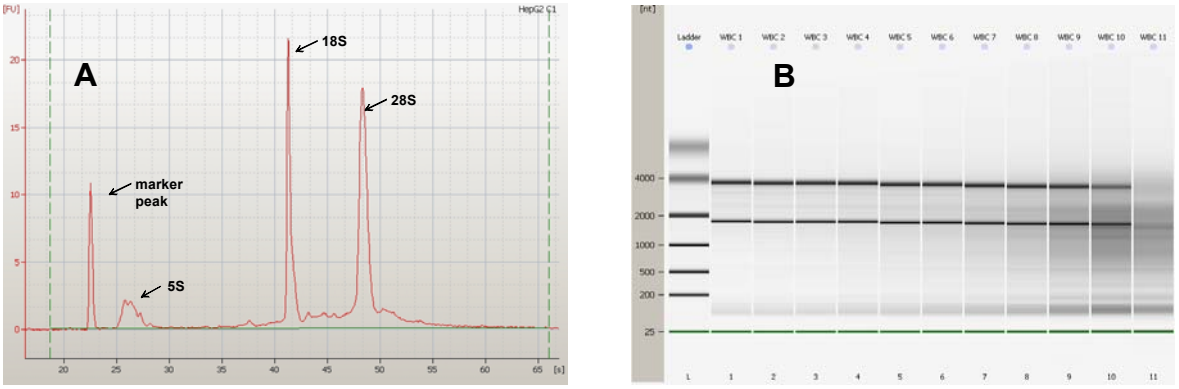

Figure 4: Electropherogram (A) and gel-like image (B) generated by the Agilent 2100 Bioanalyzer Software

Additionally, to simplify the assessment of RNA integrity the instrument software calculates a numerical value: RNA integrity number (RIN) on the 2100 Bioanalyzer and RNA quality index (RQI) on the Experion. A RQI/RIN of 1 represents almost fragmented and degraded RNA and a RQI/RIN of 10 represents intact and non fragmented RNA [35].

The evaluation of RNA integrity should also be integrated as a routine step in new applications like the expression profiling of miRNAs, as little is known about the accessibility of miRNA to degradation and the influence of total RNA integrity as a factor possibly compromising the expression profiling of miRNAs [40]. As miRNAs belong to the group of nucleic acids, they are examined with the same technologies as long RNAs e.g. mRNAs. Problems start with the quantification and quality control of miRNAs, as classical photometrical methods for measuring the concentration of nucleic acids do not allow discriminating between different fractions of RNAs. Agilent Technologies offers a new small RNA tool on the 2100 Bioanalyzer making it possible to analyze small RNA (< 200 nt) with the lab-on-chip technology. Within this small RNA fraction, fragments with a size of 15-40 nt are defined as miRNA (figure 5). The concentration of miRNA is calculated as absolute

concentration [pg/µl] and as a percentage of small RNA [%]. By now, this chip offers one of the few possibilities to selectively quantify miRNA.

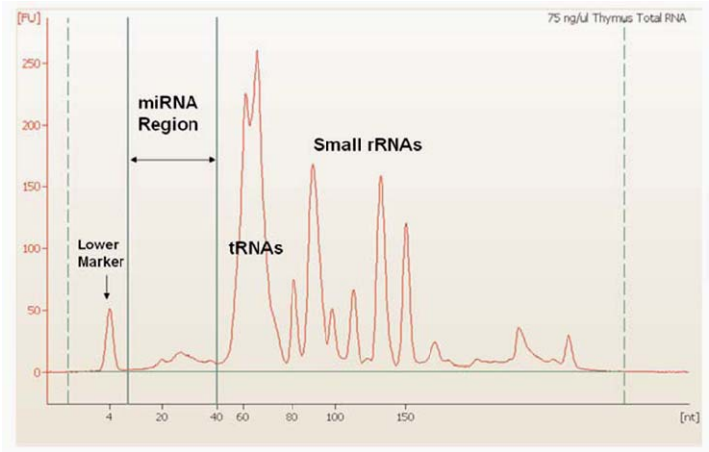

Figure 5: Image of a typical electropherogram for small RNA analysis performed with the Small RNA Assay on the 2100 Bioanalyzer
(http://www.chem.agilent.com/Library/technicaloverviews/Public/5989-7002EN.pdf)

1.5 Aim of the thesis

The objective of this thesis was to investigate, if transcriptomics (mRNA and miRNA) are suitable to discover the illegal application of anabolic steroids (figure 6). Few is known about transcriptional changes in primary and secondary hormone-dependent target tissues of female cattle. Hence, it was the aim to reveal mRNA expression changes in reproductive organs and liver induced by anabolics. Additionally, this work aimed to focus on the influence of steroid hormones on miRNA expression profiles, as this had never been investigated by now. The final scientific objective was to test with appropriate bio-statistical tools, if gained gene expression results could be combined to a gene expression pattern, which might be suitable to be used as biomarker for the surveillance of anabolic misuse.

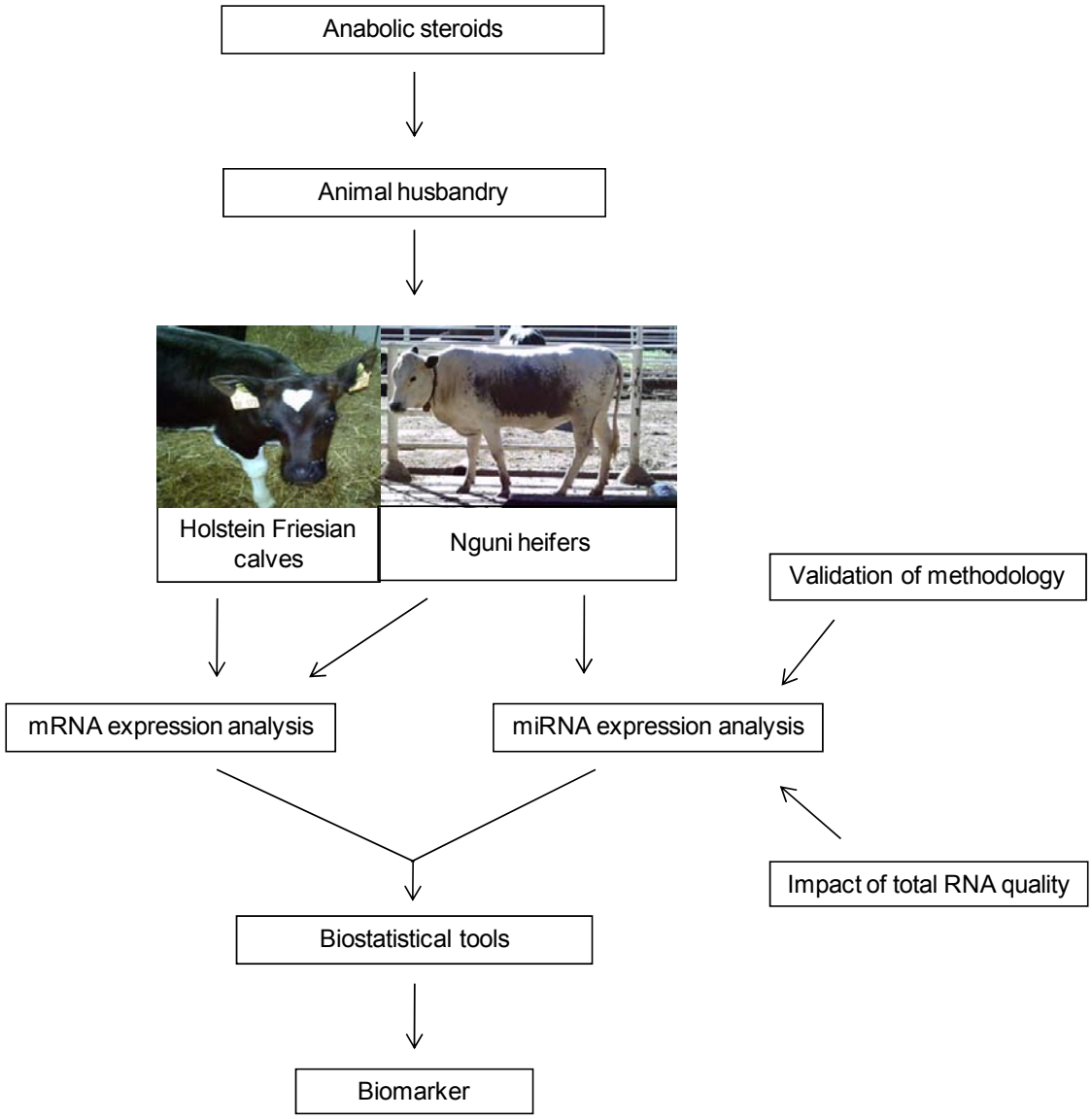

Figure 6: Schematic overview representing the aims of the thesis

2 Material and methods

2.1 Collection of biological sample material

2.1.1 Animal studies

South Africa study on Nguni heifers

18 healthy, post-pubertal, non-pregnant, 2 years old Nguni heifers were divided into a control and a treatment group of nine animals each. The animal attendance was done by the Onderstepoort Veterinary Institute (Onderstepoort, Pretoria, South Africa). The treatment group was implanted with Revalor H, an anabolic preparation licensed in several countries including the Republic of South Africa and containing 140 mg TBA plus 20 mg E2 (Intervet, Spartan, South Africa) according to the manufacturer´s instructions into the middle third of the pinna of the ear for 42 days. Plasma samples for analysis of progesterone levels have been taken during the course of the trial 11 and 5 days before treatment and on day 2, 7, 9, 16, 22, 24, 29, 32 and 39 after the application of the anabolic preparation. Plasma progesterone levels have been determined using an enzyme immunoassay (EIA) as previously described [41].

At slaughter, tissue samples (liver, uterine endometrium, ovary) were collected. Tissue was cut into small pieces and conserved in RNA*later* (Applied Biosystems, Darmstadt, Germany) immediately after the removal and stored at -80°C until further analysis.

Pour on anabolics study on pre-pubertal Holstein Friesian calves

20 pre-pubertal, female, 2 weeks old Holstein Friesian calves with a mean body weight of 61.4 ± 5.73 kg were randomly assigned to four groups of five animals each. Group 1 remained untreated and served as control. Animals of group 3 and group 4 were treated once or three times in weekly intervals, respectively, with a hormone mix containing 25 mg estradiol benzoate (Sigma Aldrich, Zwijndrecht, The Netherlands), 60 mg testosterone decanoate (Sigma Aldrich) and 60 mg testosterone cypionate (Sigma Aldrich). The hormone mix was applied in two different ways: per intra muscular injection (one animal per group) or via pour on treatment (four animals per group). For pour on treatment, animals were shaved on the back from neck to tail and 10 mL of the hormone mix were rubbed onto the skin. Four substances served as carrier solvents for the pour on treatment to ensure the transit of the hormone mix from the skin into the organism: Ivomec (Spruyt Hillen, IJsselstein, The Netherlands), dimethyl sulfoxide (DMSO) (Spruyt Hillen), Miglyol 840 (Spruyt Hillen) and diethylen glycol monobuthyl ether (DEGMBE) (Spruyt Hillen). For injection, Arachide oil (Spruyt Hillen) was used. Group 2 received only the carrier substances without the hormone

mix three times in weekly intervals to serve as a carrier control group. The treatment pattern is displayed in table 1. Animals were slaughtered 92 days after the beginning of the experiment.

Table 1: Treatment scheme of the "pour on anabolics" study

Group	Animal	Treatment	Group	Animal	Treatment
Control	1	none	1x	11	Injection of hormone mix, 1x
	2	none		12	Degmbe + hormone mix, 1x
	3	none		13	DMSO + hormone mix, 1x
	4	none		14	Ivomec + hormone mix, 1x
	5	none		15	Miglyol + hormone mix, 1x
Carrier Control	6	Oil inject, 3x	3x	16	Injection of hormone mix, 3x
	7	Degmbe, 3x		17	Degmbe + hormone mix, 3x
	8	DMSO, 3x		18	DMSO + hormone mix, 3x
	9	Ivomec, 3x		19	Ivomec + hormone mix, 3x
	10	Miglyol, 3x		20	Miglyol + hormone mix, 3x

Animal attendance was done according to practice and the treatment protocol has been approved by the ethical committee of the "Regierung von Oberbayern".
At slaughter, tissue samples (ovary) were collected. Tissue was cut into small pieces and conserved in RNA*later* (Applied Biosystems) immediately after the removal and stored at -80°C until further analysis.

2.1.2 Tissue and blood collection for methodological investigations

Tissue samples (liver, kidney, heart, intestine and muscle) (n=6) for the validation of miRNA methodology and for the investigation of the influence of total RNA quality on gene expression analysis were obtained from the slaughterhouse in Landshut (Germany). Tissue was cut into small pieces and conserved in RNA*later* (Applied Biosystems) immediately after the removal and stored at -80°C until further analysis.
Blood samples were taken from cows of the experimental station Veitshof (Freising, Germany). Whole blood (9 mL) was collected and stabilized in EDTA vacutainers (Greiner Bio-One, Frickenhausen, Germany).

2.2 Purification of ribonucleic acids (RNA)

Validation of miRNA extraction

Two different kits, which were optimized for the preservation of small RNA species in the extraction process, were tested: the *mir*Vana Kit (Applied Biosystems) and the miRNeasy Mini Kit (Qiagen, Hilden, Germany). At first, both kits were applied in the in-house called "two-tube-extraction" method. In this manner, after homogenization and lysis of the tissue, a phase separation of RNA, DNA and protein was done using phenol (Qiagen) and chloroform (Merck, Darmstadt, Germany). The upper aqueous phase containing RNA was collected. After adjustment of the ethanol concentration to gain certain binding specificities, the RNA was bound to a silica-based membrane included in the kit. Due to the ethanol content, just the long RNAs were bound to the membrane. The flow-through containing the small RNAs is collected and bound to another membrane. After certain steps of washing, the specific RNA fractions [long RNA (>200 nt) and small RNA (<200 nt) can be eluded from the membranes.
Continuative, the miRNeasy Mini Kit was also tested in the "one-tube-extraction" method. In this way, after the homogenization and lysis, the ethanol concentration was adjusted to bind total RNA on the silica-based membrane. Herein, after washing and elution, one total RNA fraction was received. The schematical procedure of the "one-tube" versus the "two-tube" extraction is displayed in figure 7. All methods were performed according to the manufacturer´s recommendations.

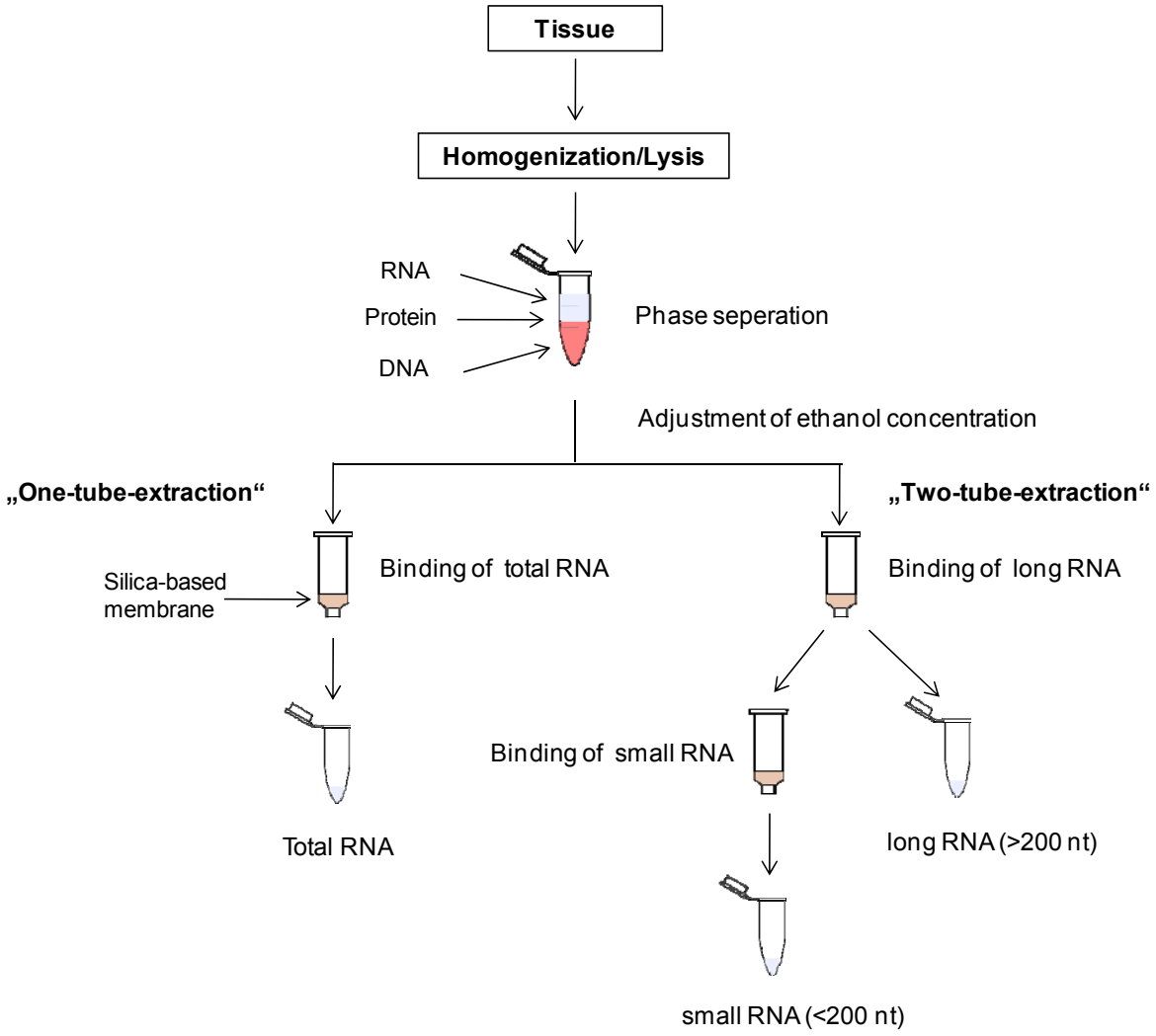

Figure 7: Schematical procedure showing the differences between the “one-tube” and the “two-tube” extraction method using the miRNeasy Mini Kit (Qiagen)

Total RNA extraction

Total RNA from tissue samples for physiological studies and for RNA quality studies were extracted from 50 mg of tissue with the “one-tube-extraction” method using the miRNeasy Mini Kit (Qiagen).

White blood cells (WBC) were extracted from whole blood samples after lysis as previously described by Hammerle-Fickinger et al. [42]. Instead of TriFast Lysis reagent (Invitrogen, Karlsruhe, Germany), Qiazol Lysis reagent (Qiagen) was used. Lysed samples were subjected to “one-tube-extraction” using miRNeasy Mini Kit (Qiagen).

RNA concentration was measured after extraction using the NanoDrop (peqLab Biotechnologie GmbH, Erlangen, Germany). $OD_{260/230}$ and $OD_{260/280}$ ratios were checked considering sample purity.

2.3 RNA degradation study

To investigate the influence of RNA degradation on small RNA quantification and gene expression analysis a degradation study was conducted. For artificial RNA degradation, RNA stock solutions of six replicates of three different tissues (liver, muscle, WBC) were pooled and the pool divided into two equal portions. One portion was degraded by exposure to UV light for 90 min to create a fragmented and degraded RNA fraction. The second portion remained untreated and served as intact and non-degraded RNA fraction [34]. To create a linear gradient between intact RNA and degraded RNA from the identical transcriptome, the two fractions were mixed in the respective ratios (table 2).

Table 2: Generation of an artificial degradation gradient with 11 steps

degradation step	intact RNA [%]	degraded RNA [%]
1	100	0
2	90	10
3	80	20
4	70	30
5	60	40
6	50	50
7	40	60
8	30	70
9	20	80
10	10	90
11	0	100

A serial dilution with 11 degradation steps was created, whereby step 1 being intact RNA (consisting of 100% intact RNA, 0% fragmented RNA) decreasing the intact RNA content in 10% steps to step 11 being the most degraded RNA (consisting of 0% intact RNA; 100% fragmented RNA). This was done for all three tissues separately.

2.4 Analysis of total RNA integrity and small RNA quantification

Control of RNA integrity and small RNA quantification were performed with the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). For RNA integrity analysis, Eukaryote Total RNA Nano Assay (Agilent Technologies) was taken and the RIN served as RNA integrity parameter. The RIN number is calculated based on a numerical system from 1 to 10, with 1 being most degraded and 10 being most intact [43].

The Small RNA Assay (Agilent Technologies) was used for quantification of small RNAs as well in the enriched small RNA fraction of the “two-tube extraction” as in the total RNA fraction of the “one-tube-extraction”.

2.5 Target gene selection

Target genes were selected by screening the actual literature for metabolic pathways in the target organs, which are known to be regulated by steroid hormones and could thereby be influenced by the exogenous application of anabolic hormones. Target genes, which were investigated in the target tissues, were sorted to different functional groups (table 3).

Table 3: Target genes measured in liver (L), uterine endometrium (U) and ovary (O_H) of Nguni heifers and ovary of Holstein Friesian calves (O_C) [12; 20; 21; 44-68]

Functional group	Gene	
Hormone receptors	Androgen receptor (AR)	L, U, O_H,O_C
	Estrogen receptors (ERα, ERβ)	L, U, O_H,O_C
	Growth hormone receptor (GHR)	L, U
	Progestin receptor (PR)	U, O_H,O_C
	Insulin-like-growth factor-1 receptor (IGF-1 R)	L, U, O_H,O_C
	Follicle stimulating hormone receptor (FSHR)	U, O_H,O_C
	Luteinizing hormone receptor (LHR)	O_H,O_C
	Vascular endothelial growth factor receptors (FLK-1, FLT-1)	O_H,O_C
	Bone morphogenetic proteins receptors (BMPR II, ALK-5, ALK-6)	O_H,O_C
	FAS receptor (FAS)	U
	Insulin receptors (IRα, IRβ)	L
	Glucocorticoid receptor α (GRα)	U, O_H
Angiogenesis	Vascular endothelial growth factor A (VEGFA)	U
	Vascular endothelial growth factors (VEGF 120, VEGF 164)	U, O_H,O_C
	Matrix metalloproteinase 2 (MMP-2)	O_H,O_C
	Matrix metalloproteinase 23B (MMP-23B)	O_C
	Tissue inhibitior of matrix metalloproteinase 2 (TIMP-2)	U, O_H,O_C
	Angiopoietins (ANGPT-1, -2)	U, O_H,O_C
	Thrombospondin (THBS)	U
	Fibroblast growth factors (FGF-1, -2, -7)	U, O_H,O_C
Trancription factors/ proliferation	Insulin-like-growth factor 1 (IGF-1)	L, U, O_H,O_C
	Insulin-like-growth factor 2 (IGF-2)	L, O_H,O_C
	IGF binding protein 1 (IGFBP-1)	L
	IGF binding protein 2 (IGFBP-2)	L, O_H,O_C
	IGF binding proteins 3 (IGFBP-3)	U, O_H,O_C
	Myelocytomatosis cellular oncogen homolog (c-myc)	L, U, O_H
	Myeloblastosis viral oncogene homolog (v-myb)	L
	c-fos transcription factor (c-fos)	U, O_H,O_C
	c-jun transcription factor (c-jun)	U, O_H,O_C
	Retiboblastoma-1 (RB-1)	O_C
	Retiboblastoma binding protein-1 (RBBP-1)	U
	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (c-kit)	O_C
	p53 tumor suppressor gene	U, O_H,O_C
	Bone morphogenetic proteins (BMP-2,-4)	U, O_H,O_C
	Bone morphogenetic protein 15 (BMP-15)	O_C
	Hepatocyte growth factor (HGF)	L
	Cyclin D2 (CYL D2)	O_C
	CCAAT/enhancer binding proteins (CEBPA,CEBPB, CEBPD)	L, U

Table 3 *(continued)*

Functional group	Gene	
Apoptosis	B-cell CLL/lymphoma 2 (bcl-2)	O_H,O_C
	B-cell leukemia/lymphoma x (bcl-xl)	U, O_H,O_C
	Caspase 3 (CASP3)	U, O_H
	Caspase 8 (CASP8)	U
	Prohibitin (PHB)	U
	FAS ligand (FASL)	U
Immune factors	Transforming growth factor β (TGFβ)	L, U, O_H,O_C
	Tumor necrosis factor α (TNFα)	L, U, O_H,O_C
	Complement components (C3, C7)	U
Cholesterol metabolism	3-hydroxy-methylglutaryl-coenzym-A-Synthase (HMGCoA-S)	L
	3-hydroxy-methylglutaryl-coenzym-A-Reductase (HMGCoA-R)	L
	Farnesyldiphosphat-farnesyltransferase (FDFT)	L
	Sterol regulatory element binding proteins (SREBP-1, SREBP-2)	L
	SREBP cleavage activating protein (SCAP)	L
Lipid metabolism	Apolipoproteins (APOA1, APOC2)	L
	Hepatic lipase (LIPC)	L
	Lipoprotein lipase (LPL)	L
Protein metabolismus	Cathepsins (CTSB, CTSL)	L, U
	Calpastatin (CAST)	U
	Tyrosine amino transferase (TAT)	L
Glucose metabolism	Lactatdehydrogenase A (LDHA)	U
	Hexokinase 1 (HK1)	U
Steroid metabolism	3β-hydroxy steroid dehydrogenase 3 (HSD3B1)	O_C
	17β-hydroxy steroid dehydrogenases (HSD17B1, HSD17B2, HSD17B3, HSD17B4, HSD17B8, HSD17B11)	L, O_H,O_C
	Cytochrome P450 11A1 (CYP11A1)	L, O_C
	Cytochrome P450 19A1 (CYP19A1)	U, O_H,O_C
	5α-Reductase (S5A1)	O_H,O_C
	Steroid hormone binding globulin (SHBG)	L
	Steroidogenic acute regulatory protein (STAR)	L, O_C
Others	Cyclooxygenase 2 (COX-2)	U
	Growth differentiation factor 9 (GDF-9)	O_H,O_C
	Smad2	O_H,O_C
	Inhibin A (INHA)	O_H,O_C
	Lactotransferrin (LTF)	U

2.6 Primer design

Primer pairs (table 4) were either newly designed using published bovine nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or Ensemble (http://www.ensembl.org/index.html), or previously established primer sequences were used [21; 23; 49; 69]. Newly designed primers were ordered and synthesized at Eurofins MWG (Ebersberg, Germany).

miScript Primer Assays for single assay miRNA expression analysis were sythesized and ordered at Qiagen. As no specific bovine primer assays are available, human assays were used after checking sequence homology using http://www.mirbase.org/.

Table 4: Primer pairs of all investigated target genes in alphabetical order with their annealing temperature (T_M), product length [base pairs (bp)] and accession number

Gene		sequence [5´→ 3´]	T_M[°C]	product lenght [bp]	Accession No.
ACTB	for	AAC TCC ATC ATG AAG TGT GAC	60	202	AY141970
	rev	GAT CCA CAT CTG CTG GAA GG			
ALK-5	for	CAG GGA AGA ACG TTC ATG GT	60	128	AF317296
	rev	CCA ACC AAA GCT GAG TCC AT			
ALK-6	for	GCC TGT TGT CAC CTC TGG AT	60	106	Z23143
	rev	CCT TTC TGT GCA GCA TTC AA			
ANGPT-1	for	TCG GAG ATG GCT CAG ATA CAG	60	229	AF093573
	rev	CCA GCA GTT GTA TTT CAA GTC GA			
ANGPT-2	for	AAT TCA GTT CTC CAA AAG CAG C	60	216	NM_001098855
	rev	TCC ACC CGT TTC CAT GTC			
ANGPT-2	for	TTA TTC AGC GAC GTG AAG ACG G	62	187	NM_001098855
	rev	TAC AGC GAG TAA GCC TGA TT			
APOC2	for	GGG TTT CTC ATC CTC CTG GT	63	215	NM_001102380
	rev	AAT CCC TGC ATA GGT GGT CAc			
APOA1	for	TTT GGG AAA ACA GCT CAA CCt	63	240	NM_174242
	rev	GCC ACT TCT TCT GGA ACT CG			
AR	for	CCT GGT TTT CAA TGA GTA ACC GCA TG	60	172	AY862875
	rev	TTG ATT TTT CAG CCC ATC CAC TGG A			
bcl-2	for	ATG ACT TCT CTC GGC GCT AC	62	245	XM_586976
	rev	CCG GTT CAG GTA CTC GGT CA			
bcl-xl	for	GGC ATT CAG CGA CCT GC	60	203	AF245487
	rev	CC TCC AAG TTG CGA TCC			
BMP-2	for	CAG TAG GTG GGA GAG CTT CG	60	194	NM_0010099141
	rev	TGA CAA GCA AGG GCC TTA TCT GT			
BMP-4	for	GAG CTT CCA CCA CGA AGA AC	60	179	NM_001045877
	rev	TAC GAT GAA GC CCT GT CCC			
BMP-15	for	GCA GGC AGT ATT GCA TCT GAA G	60	250	NM_001031752
	rev	CAC TCT GAT CCA CCA GCT AC			
BMPR II	for	CAA AGA TTG GCC CTT ATC CA	60	109	AJ534390
	rev	CTG GAC ATC GAA TGA TCT GA			
C3	for	ATT GCC AGG TTC TTG TAC GGG	60	258	NM_001040469
	rev	GTC ACT GCC TGA TTG CAA GAT G			
C7	for	GGC GGT CAA TTG CTG TTT ATG G	60	232	NM_001045966
	rev	GGT CTG CTT TCT GCA TCC TC			
c-fos	for	CAG TGC CAA CTT CAT CCC AAC	60	189	NM_182786
	rev	CTG CCT CCT GTC ATG GTT TTC			
c-jun	for	CGG CTA TAA CCC CAA GA	60	243	AF_069514
	rev	CCT GCT CAT CTG TCA CGT TC			
c-myc	for	TCT TGC GCC TAA ATT GAC CTA TTG	60	153	ENSBTAT00000011066
	rev	GCC AAG GTT GTG AGG TTG TTC			
c-kit	for	AAG TCC ATG CTG TCG AAG AA	60	185	XM_612028
	rev	TCT GCT GGC TGT TTT CCT TTA			
CASP3	for	GAC AGT GGT GCT GAG GAT GA	60	164	NM_001077840
	rev	CTG TGA GCG TGC TTT TTC AG			
CASP8	for	TAG CAT AGC ACG GAA GCA GG	62	294	DQ319070
	rev	GCC AGT GAA GTA AGA GGT CAG			
CAST	for	GAT CAG AAG TGC TGC TCC A	60	206	NM174003
	rev	GGA CTG TTT CCT CAT CTT ACC			
CEBPA	for	CCA AGA AGT CCG TGG ACA AG	62	184	NM_176784
	rev	AGT TCG CGG CTC AGT TGT TC			

Table 4 *(continued)*

Gene		sequence [5´→ 3´]	T_M[°C]	product lenght [bp]	Accession No.
CEBPB	for	GCA CAG CGA CGA GTA CAA GA	60	152	NM_176788
	rev	GTT GCT CCA CCT TCT TCT GG			
CEBPD	for	ATC GAC TTC AGC GCC TAC ATC	62	101	BC133581
	rev	GCT TTG TGG TTG CTG TTG AAG AG			
CTSB	for	GAT CTG CAT CCA CAG CCA	60	192	NM174031
	rev	ATG GAG TAC GGT CTG CAA CC			
CTSL	for	TCC ATA TCT TGC AAC GGA CAC TTT A	60	110	NM174032
	rev	CCT TCA TAA GGG CCT TCT CC			
COX 2	for	GCC AGG GGA GCT ACG ACT A	60	247	NM_174445.2
	rev	AAG GAC AAT GGG CAT GAA ACT GTG			
CYL D2	for	TGC AGA ACT TGC TGA CCA TCG	57	171	BC120199
	rev	GGT AAT TGA TGG CGA GAG GAA AG			
CYP11A1	for	CGG AAA GTT TGT AGG GGA CAT C	62	177	NM_176644
	rev	ACG TTG AGC AGA GGG ACA CT			
CYP19P1	for	TCA ACA GC GAG AG CTG GAA G	62	181	NM_174305
	rev	GGG GAT GCT TTG CAA TAA GAA ACA			
ERα	for	AGG GAA GCT CCT ATT TGC TCC	60	234	AF177936
	rev	CGG TGG ATG TGG TCC TTC TCT			
ERβ	for	GAG ATA TTC TTT GTG TTG GAG TTT	60	164	NM_174051
	rev	CTT CGT GGA GCT CAG CCT GT			
FAS	for	TGT TGT CAG CCT TGT CCT CC	60	174	U34794
	rev	GTT CCA CTT CTA GCC CAT GTT C			
FASL	for	CAT CTT TGG AGA AGC AAA TAG	60	205	AB035802
	rev	GGA ATA CAC AAA ATA CAG CCC			
FDFT	for	GAA ATG CGC CAT GCA GTA	60	198	NM_001013004.1
	rev	GGA GAT CGT TGG GAA GTC CT			
FGF-1	for	TTG TAC GGC TCA CAG ACA CC	60	169	NM_174055
	rev	CTT TCT GGC CGA TGT GAG TC			
FGF 2	for	AGC CTT GCA ACT CTG CTT GT	60	210	NM_174056.3
	rev	CGA ATT CAG ATC CCT CCT GA			
FGF-7	for	GAC ATG GAT CCT GCC AAG TT	60	129	XM_869016
	rev	GGG CTG GAA CAG TTC ACA TT			
FLK-1	for	GCT TCT ACC AGG ACA CTG ACA T	60	144	X94298
	rev	AAC ACG GAA TCA CCA CCA CAG TT			
FLT-1	for	ATG ACC GAA GGG AAG AAG GTG	60	193	XM_001249768
	rev	TGA CTG TTG TCT CGC AGG TC			
FSHR	for	AGTTGCCCTTTTTCCCATCTTTGG	64	150	NM_174061.1
	rev	TAGCAGCCACAGATGACCACAA			
GDF-9	for	CAT CGG TAT GGC TCT CCA GT	60	122	NM_174681
	rev	ATG GCC AAA ACA CTC AAA GGA CT			
GHR	for	CCA GTT TCC ATG GTT CTT AAT TAT	60	136	NM176608
	rev	TTC CTT TAA TCT TTG GAA CTG G			
GRα	for	TTC GAA AAA ACT GCC CAG C	62	194	AY238475
	rev	CAG TGT TGG GGT GAG TTG TG			
H3	for	ACT GCT ACA AAA GCC GCT C	60	233	NM_001014389
	rev	ACT TGC CTC CTG CAA AGC AC			
HGF	for	GAT GTC CAT GGG AGA GGA GA	62	170	NM_001031751
	rev	TCA GGA ATT GTG CAC CCA TAA TTA G			
HK1	for	CAA GAC GCA CCC ACA GTA TCC	60	211	NM001012668
	rev	TCA CCT CCA GCA GCA TTT CCT T			

Table 4 *(continued)*

Gene		sequence [5´→ 3´]	T_M[°C]	product lenght [bp]	Accession No.
HMGCoA-R	for	CTC TCT AAA ATG ATC AGC AT	60	132	
	rev	TCA ACT TTT CTT TCT CTG TTT			
HMGCoA-S	for	GAT GGT CGC TAT GCA CTG GT	60	246	NM_001045883.1
	rev	GCC CTC TCT CGA GGA CCA GA			
HSD3B1	for	TCC ACA CCA GCA CCA TAG AA	57	178	NM_174343
	rev	AAG GTG CCA CCA TTT TTC AGA G			
HSD17B1	for	CTC ATT ACC GGC TGT TCC TC	57	200	NM_001102365
	rev	ATG GAA TCT GCA TCC CTC ACg			
HSD17B2	for	CAT CTC AGG CAC GAG TCA AAT G	62	125	NM_001075726.1
	rev	CAC TGG GGA GAT GTC TGG AT			
HSD17B3	for	CCC AAG CCA TTT CCT TAA CAC G	60	198	BC109700
	rev	ACA AAA GCC TTG GAA GCT GAA TAC			
HSD17B4	for	CGG ATG ACC CAA AGC ATT TTG C	60	176	NM_001007809.1
	rev	TCT GTC TCA CAA GGG CTC CAA			
HSD17B8	for	GGG CAT CAC CAG AGA TGA AT	60	228	NM_001046324
	rev	CAA TCA CTC CAG CCT TGG AT			
HSD17B11	for	GGT GAA GGC AGA AGT TGG AG	62	228	NM_001046286
	rev	AAG AAG GGG ACC CCA GTA TG			
IGF-1	for	CAT CCT CCT CGC ATC TCT TC	60	239	NM_001077828
	rev	CTC CAG CCT CCT CAG ATC AC			
IGF-2	for	ACC CTC CAG TTT GTC TGT GG	54	166	BC126514
	rev	ACA CAT CCC TCT CGG ACT TG			
IGFBP-1	for	ACC AGC CCA GAG AAT GTG TC	64	244	NM_174554
	rev	GTT TGT CTC CTG CCT TCT GC			
IGFBP-2	for	AGC ATG GCC TGT ACA ACC TC	60	157	NM_174555
	rev	CCC TGC TGC TCG TTG TAG AA			
IGFBP-3	for	ACA GAC ACC CAG AAC TTC TCC T	60	202	NM174556
	rev	AGA AAC CCC GCT TCC TGC C			
IGF-1 R	for	CCC AAA ACC GAA GCT GAG AAG	60	314	X54980
	rev	CAT CCT CCT CGC ATC TCT TC			
IL-1β	for	TTC TCT CCA GCC AAC CTT CAT T	60	198	M37211
	rev	ATC TGC AGC TGG ATG TTT CCA T			
INHA	for	TAG TGC ACC CTC CAA GTT TC	60	239	NM_174094
	rev	GGT TGG GCA TCT CAT AC			
IRα	for	TCC TCA AGG AGC TGG AGG AGT	62	89	AJ320235
	rev	TTT CCT CGA AGG CCT GGG GAT			
IRβ	for	TCC TCA AGG AGC TGG AGG AGT	60	111	AJ320235
	rev	TAG CGT CCT CGG CAA CAG G			
LDHA	for	GTG GCT TGG AAG ATA AGT GG	60	155	NM174099
	rev	ACT AGA GTC ACC ATG CTC C			
LHR	for	CAG TGT GCT CCT GAA CCA GA	60	192	NM_174381
	rev	GTC TGC AAA GGA GAG GTT GC			
LIPC	for	CGC CAT TCA CAC CTT TAC CT	62	186	NM_001035410
	rev	TCA TGG GCA CAT TTG ACA GT			
LPL	for	CTC CTG ATG ATG CGG ATT TTG TA	62	196	NM_001075120
	rev	ACC AGC TGA TCC ACA TCT CC			
LTF	for	ACC ATC TCC CAA CCT GAG TG	60	285	NM_180998
	rev	AAA GTT GCT GCC CTT CTT CAC G			

Table 4 *(continued)*

Gene		sequence [5´→ 3´]	T_M[°C]	product lenght [bp]	Accession No.
MMP-2	for	CCC AGA CAG TGG ATG ATG C	60	237	NM_174745
	rev	TTG TCC TTC TCC CAG GGT C			
MMP-23B	for	CGC GCT ACA GCT GGA AGA AAG GC	62	163	NM_001038556
	rev	ACA GCT CGT CCT GCG ATA GT			
p53	for	ATT TAC GCG CGG AGT ATT TG GAC	60	174	NM_174201.2
	rev	CCAGTGTGATGATGGTGAGGA			
PHB	for	GTG AGC GAT GAC CTC ACA GA	60	163	NM_001034572.1
	rev	CAG CCT TTT CCA CCA CAA ATC T			
PR	for	ACC AGC CCT ATC TCA ACT ACC	60	186	XM_583951.4
	rev	TAT GCT GTC CTT CCA TTG CCC			
RB-1	for	CAA ATT CAG AGG CAC AAG CAA	62	179	NM_001076907
	rev	CTG GAA AAG GGT CCA GAT GAT			
RBBP-1	for	TTT CCA GGT CCA CTG GTC TC	60	226	NM_001034638
	rev	CTC AGA CAC CGA GCA AAT GAC			
S5A1	for	CCT TCC TAT TGG CGT TCA TCT TC	62	180	NM_001099137
	rev	ATT CAA ACA AGC CCC CTC TTG GT			
SCAP	for	GGT CAC TTT CCG GGA TGG	60	179	NM_001101889.1
	rev	TGG GTA GCA GCA GGC TAA GA			
SHBG	for	ACT TGG GAT CCA GAG GGA GT	62	188	NM_001098858
	rev	TCC CCA TGG ATC TTC ACT TCc			
SREBP-1	for	CCA GCT GAC AGC TCC ATT GA	60	67	NM_001113302.1
	rev	TGC GCG CCA CAA GGA			
SREBP-2	for	CAG GTC CTG GTA CAG CCT CA	60	158	
	rev	GCT CTT ACC GGA ACT TGC AG			
Smad2	for	ATG GTC GTC TTC AGG TGT CC	60	237	NM_001046218
	rev	GCA GTT CCG TTA GGA TCT CG			
STAR	for	TGG AAA AGA CAC GGT CAT CA	57	154	NM_174189
	rev	CTG GGG CAT CTC CTC ATA GA			
TAT	for	ACC CTT GTG GGT CAG TGT TC	60	167	BT021798
	rev	ACA GGA TGG GGA CTT TGC TG			
TGFβ	for	ACG TCA CTG GAG TTG TGC GG	63	267	XM5929497
	rev	TTC ATG CCG TGA ATG GTG GCG			
THBS	for	ACA CGA CTG CAA CAA GAA CGC	62	199	NM_174196
	rev	GGT TGG GGC AAT TAT CCT TTG T			
TNFα	for	CCA CGT TGT AGC CGA CAT C	63	197	NM173966
	rev	CCC TGA AGA GGA CCT GTG AG			
VEGFA	for	GGT GGA CAT CCT CCA GGA GTA	60	177	NM_174216.1
	rev	CTA TGT GCT GGC TTT GGT GAG			
VEGF 120	for	CCG TCC CAT TGA GAC CCT G	60	296	AB455252
	rev	CGG CTT GTC ACA ATT TTT CTT GTC			
VEGF 164	for	CCG TCC CAT TGA GAC CCT G	62	278	
	rev	GCC CAC AGG GAT TTT CTT GC			
UBQ	for	AGA TCC AGG ATA AGG GAA GGC AT	60	198	Z18245
	rev	GCT CCA CCT CCA GGG TGA T			
v-myb	for	TCA CGT CCC ATA TCC TGT AGC	62	170	NM_175050
	rev	CCT GTC CTT TGA GTT CGT TCT CA			

2.7 cDNA synthesis

2.7.1 cDNA synthesis for mRNA expression analysis

RNA samples have been converted to cDNA using M-MLV H^- reverse transcriptase (Promega, Regensburg, Germany). Therefore, 500 ng of total RNA were diluted to a final volume of 13 µL. The mastermix for the reverse transcription (RT) was prepared as follows: 4 µL 5 x reaction buffer (Promega), 1 µL random hexamer primers (Invitrogen), 1 µL dNTPs (Fermentas, St. Leon-Rot, Germany), 1 µL M-MLV H^- reverse transcriptase (Promega). After adding 7 µL of the mastermix to the diluted RNA, the plate was inserted in the Eppendorf Gradient Mastercyler (Eppendorf, Hamburg, Germany) and the here stated temperature protocol was started: 21°C, 10 min; 48°C, 50 min; 90°C, 2 min; 4°C hold. Finally, cDNA samples were diluted with 40 µL of RNAse free water (5Prime, Hamburg, Germany) to a final volume of 60 µL. RT was done in duplicates for every sample.

2.7.2 cDNA synthesis for miRNA expression profiling using PCR arrays

For cDNA synthesis from miRNA, total RNA samples were poly-adenylated, elongated and reverse transcribed using the miRCURY Universal cDNA Sythesis Kit (Exiqon, Vedbaek, Denmark). 25 ng of total RNA were diluted to a final volume of 14 µL, which was added to 4 µL 5x reaction buffer plus 2 µL enzyme mix. In the Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany), the reaction mix was incubated at 42°C for 60 min followed by a heat-inactivation of the enzyme at 95°C for 5 min and a hold step at 4°C.

2.7.3 cDNA synthesis for miRNA expression analysis using single assay PCR

For single assay PCR, samples were poly-adenylated, elongated and reverse transcribed using the miScript System (Qiagen). For a final volume of 10 µL, 2 µL 5 x miScript buffer plus 1 µL miScript reverse transcriptase were mixed with 500 ng of total RNA, which were diluted to a volume of 7 µL. The reaction was pipetted in a 96-well plate and the plate was inserted in the Eppendorf Gradient Cycler (Eppendorf) and the here stated temperature protocol was started: 37°C, 60 min; 95°C, 5 min; 4°C hold. After RT, all samples were diluted 1:6 to a final volume of 60 µL.

2.8 Quantitative PCR (qPCR)

2.8.1 mRNA expression analysis

qPCR was performed using MESA Green qPCR MasterMix plus for SYBR Assay w/ fluorescein (Eurogentec, Cologne, Germany) by a standard protocol recommended by the manufacturer. The mastermix was prepared as follows: 7.5 µL 2 x MESA Green qPCR MasterMix; 1.5 µL forward primer (10 pmol/µL); 1.5 µL reverse primer (10 pmol/µL); 3.0 µL RNAse free water (5Prime). For a total volume of 15 µL, 13.5 µL of the mastermix were filled in a well and 1.5 µL of cDNA were added. The plate was heat-sealed with the Eppendorf Heat-Sealer (Eppendorf), placed in the iQ5 Cycler (Bio-Rad, Munich, Germany) and the following PCR protocol was started: denaturation step (95°C, 5 min), cycling program (40 cycles): 95°C, 3 s; primer specific annealing temperature (see table), 60 s; and melting curve analysis.
Optimal primer annealing temperature has been examined using gradient PCR on the iQ5 with a range of the annealing temperature from 54°C-64°C. Primer specificity has been proven by checking the melting curve for one distinct peak.

2.8.2 miRNA expression analysis

2.8.2.1 miRNA expression profiling using PCR arrays

miRNA expression profiling was done using human miRCURY Ready-to-use PCR Panels (Exiqon) measuring 730 different miRNAs on two plates. For miRNA expression screening, 3 samples of the control and the treatment group, respectively, were analyzed.
2180 µL of RNase free water (Exiqon) were added to the cDNA from the RT step to a final 110x dilution. This dilution was mixed 1:1 with 2200 µL of the miRCURY SYBR Green Mastermix (Exiqon). 10 µL of the final mastermix were added to every well on the Ready-to-use Panels.
For automation, pipetting was done with the epMotion 5075 LH (Eppendorf). After sealing with the 4s2 Thermal Sealer (4titude, Berlin, Germany), the plate was inserted in the CFX Cycler (Bio-Rad) and the following temperature protocol was started: polymerase activation/denaturation step (95°C, 10 min), cycling program (40 cycles): 95°C, 10 s; 60°C, 1 min; ramp rate of cooling from 95°C to 60°C: 1.6°C/s, and melting curve analysis.

2.8.2.2 miRNA expression analysis using single assay PCR

Single assay qPCR for miRNA was done using miScript System (Qiagen) according to the manufacturer´s recommendation. The following qPCR mastermix was prepared: 10 µL 2 x QuantiTect SYBR Green PCR mastermix, 2 µL 10 x universal primer, 2 µL 10 x miScript primer assay, 4 µL RNAse free water.

18 µL of the prepared mastermix were filled in a well and 2 µL template from miRNA RT for single assay PCR were added for a total volume of 20 µL. qPCR was conducted in 96-well plates, which were sealed with the Eppendorf Heat-Sealer (Eppendorf) and the following PCR temperature protocol was started in the Realplex cycler (Eppendorf): denaturation step (95°C, 15 min), cycling program (40 cycles): denaturation (95°C, 15 s), annealing (55°C, 30 s), elongation (70°C, 30 s) and melting curve analysis.

2.8.2.2.1 Sequencing of miRNA PCR products

As human miRNA primers were used for bovine miRNA amplification, it should be controlled if specific products were amplified in qPCR, even if sequences show no 100% homology between human and bovine.

Purification of miRNA PCR products

For purification of miRNA PCR products from single assay qPCR, MinElute PCR purification Kit (Qiagen) has been used according to the manufacturer´s instructions. This kit is optimized for the purification of PCR products with a length of 70 bp-4 kbp.

Concentration of the purified product has been measured using the NanoDrop (peqLab) and ds copy number calculator (http://www.uri.edu/research/gsc/resources/cndna.html) has been used to determine the copy number.

Cloning and transformation of purified PCR products

TOPO TA ® Cloning Kit for sequencing with chemically competent One Shot TOPO 10 cells (*E. coli*) (Invitrogen) was applied for cloning and transformation of the purified PCR products. The plasmid vector pCR4-TOPO, which is integrated in the kit, is linearized with a single 3´ thymidine overhang making it suitable for cloning of Taq amplified PCR products. Topoisomerase I from *Vaccina* virus is covalently bound to the vector for breaking the DNA duplex backbone.

For cloning, 10^9 copies of the purified PCR product in a total volume of 4 µL RNase free water (Qiagen) have been mixed with 1 µL Salt Solution and 1 µL TOPO vector representing a 1:3 molar ratio between the PCR product and the vector. Transformation was done

according to the instructions of the manufacturer. The transforming solution was platted on LB plates containing 50 µg/µL Kanamycin (Roth, Karlsruhe, Germany).

Colony PCR

After 24h of incubation at 37°C, grown colonies were picked from the plate and were applied to colony PCR using FastStart Taq Polymerase PCR Kit (Roche, Mannheim, Germany). The mastermix per reaction was prepared as follows: 15.2 µL RNase free water, 2 µL reaction buffer, 1.6 µL $MgCl_2$, 0.32 µL Primer (T3, T7), 0.4 µL dNTPs and 0.16 µL Taq Polymerase. One colony was added per reaction and the following temperature protocol was started: activation of Taq Polymerase (95°C for 10 min); 3-step PCR cycling (40 cycles): denaturation (95°C for 30s), annealing (55°C for 30s) and elongation (72°C for 60s); 4°C hold.

Gel electrophoresis for analyzing products from colony PCR

An 2% agarose gel (Roche) was prepared and for staining 0.2 µL GelRed (Biotium, Hayward, USA) per 10 mL gel were added. 1 µL bromphenol blue (Merck) plus 3 µL TAE buffer were mixed with 5 µL PCR product from colony PCR. 0.3 µL PCR marker (New England Biolabs, Frankfurt a.M., Germany) were used for length determination.

The gel was run for 25 min at 90 mV. Bands with a length of 200 bp were identified for successful transformation of the PCR product into the pCR-4 TOPO vector.

Positive colony PCR products were purified using MinElute PCR purification Kit (see 2.8.1). Purified products were sequenced by sequencing service at Sequiserve (Munich, Germany).

2.9 Evaluation of gene expression data

Gene expression data were analyzed using relative quantification. Data from miRNA expression profiling were initially processed using GenEx v. 5.0.2.8 (multiD Analyses AB, Gothenburg, Sweden). After merging data from all panels and inter-plate calibration, data were also subjected to relative quantification. Suitable reference genes (RG) for normalization of gene expression data were evaluated individually for all experiments using the geNorm and Normfinder algorithm in GenEx v. 5.0.2.8 (multiD Analyses AB). Optimal number of RG was selected using pairwise variation analysis integrated in geNorm algorithm implented in GenEx.

The geometric mean of two RG for physiological mRNA studies [histon 3 (H3), ubiquitin (UBQ)] and for miRNA studies (miR-122, miR-186) was used as reference index. Data were

normalized and relatively compared to the control group according to the ΔΔCq-model with the following formulas [70]:

$$\Delta Cq = Cq_{(target\ gene)} - Cq_{(reference\ gene\ index)}$$

$$\Delta\Delta Cq = \Delta Cq_{(treatment\ group)} - mean\ \Delta Cq_{(control\ group)}$$

The expression ratio of the treatment group compared to the control group is expected as $2^{-\Delta\Delta Cq}$ and represents the x-fold regulation with a value of 1 indicating no expression change after treatment.

Relative expression data were tested for normal distribution and statistically evaluated using Sigma Stat 3.0 (SPSS Inc., Chicago, IL, USA). The determined p-values for statistical significance were examined using Student´s t-test. Results with $p \leq 0.05$ were considered as statistically significant.

Linear Regression was used to determine the correlation of quantitative cycle (Cq) value and RIN value in the degradation study and the correlation between Cq values from PCR arrays and single assay PCR. Linear regresion was also used to test the linearity of the miRNA qPCR assay. Significance of linear regression was analysed by Students´s t-test by testing the slope to be different from zero. Level of significance was set for $p < 0.05$. Regression data were graphically plotted using SigmaPlot 11.0 (SSPS Inc).

To visualize the multivariate response of the selected target genes to the treatment, the method of principal component analysis (PCA) was employed using GenEx v. 5.0.2.8 (multiD Analyses AB). PCA involves a mathematical procedure that transforms a number of variables (here normalized expression values) into a smaller number of uncorrelated variables called principal components. By this, the dimensionality of the data is reduced to a number of dimensions that can be plotted in a scatter plot, here two dimensions. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible.

3 Results and discussion

3.1 mRNA studies

3.1.1 Impact of trenbolone acetate plus estradiol-17β on liver in Nguni heifers

3.1.1.1 Plasma progesterone levels

The analysis of plasma progesterone levels (figure 8) showed a decrease in the treatment group compared to the control group. Significant differences between the control and treatment group could be observed at day 2, 22, 24, 29 and 39 showing a clear initial response to the anabolic treatment and documenting the ongoing effectiveness of the anabolic preparation Revalor H till the end of the animal trial.

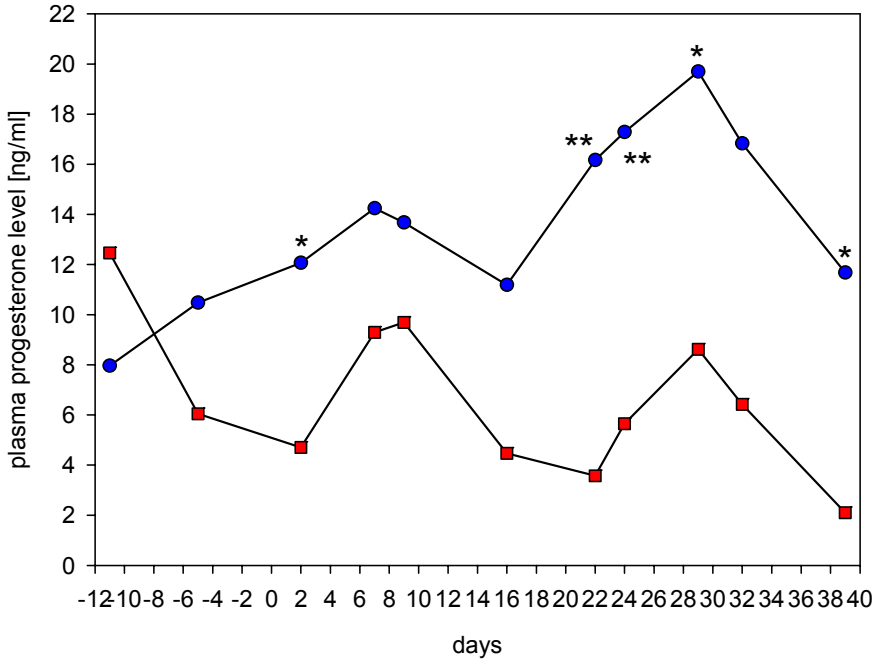

Figure 8: Plasma progesterone levels of Nguni heifers (blue circles represent control animals, red squares represent animals of the treatment group) determined during the course of the study, showing significant differences between the control group and the treatment group at day 2, 22, 24, 29, 39 (* = $p \leq 0.05$; ** = $p \leq 0.01$)

Revalor H was chosen for treatment in the actual study as it is an anabolic supplement widely used and licensed in the Republic of South Africa. Several investigations showed its efficacy in cattle and the beneficial effect on meat quality and quantity [71]. Also, the activity of this combination on day 42 has been proven, as the intended effect is persistent up to day

105 after implantation and residues of the applied drug are detectable in blood up to day 90 [72]. Also the actual measurement of the plasma progesterone levels showed a significant difference between the control and the treatment group with the most distinct differences at days 22-39 proving the effective feedback of Revalor H till the end of the experimental course. Progesterone levels were lower in the treatment group compared to the control group due to negative feedback mechanisms following the exogenous application of steroid hormones.

3.1.1.2 RNA Integrity

All 18 samples were examined using the Eukaryote Total RNA Nano Assay on the Agilent 2100 Bioanalyzer. The RNA samples showed good RNA quality with a mean RIN of 8.34 ± 0.36, which is perfectly suitable for PCR analysis [73].

3.1.1.3 Gene expression analysis

Steroid hormones are known to alter expression of various genes by acting as transcription factors and thereby mediate their physiological effect. The influence of the anabolic combination Revalor H (TBA plus E2) on gene expression in liver of Nguni heifers was investigated. Eleven out of 34 investigated genes were significantly regulated ($p \leq 0.05$) in the treatment group compared to the control group, four genes did not reach the level of significance, but a trend for regulation could be observed ($p < 0.1$). Significant regulations could be obtained for genes from the receptors group, the proliferation group, the lipid and cholesterol metabolism group, the steroid metabolism and the protein and glucose metabolism group. The x-fold regulations of the significantly regulated genes are summarized in table 5.

Table 5: Regulated target genes in liver of Nguni heifers after steroid treatment
(* = p<0.05; **= p<0.01; ***=p<0.001; value 1.00 represents no expression change)

functional group	gene	x-fold regulation		p-value	significance
		↑	↓		
receptors	AR	1.68		0.010	**
proliferation	IGF-1	1.89		0.012	*
	IGF-2		0.77	0.001	***
	IGFBP-2		0.27	0.001	***
	TNFα		0.70	0.082	
	HGF		0.78	0.072	
protein metabolism	TAT		0.61	0.028	*
	CTSB		0.77	0.009	**
lipid/cholesterol metabolism	HMGCoA-S		0.74	0.023	*
	APOC2		0.75	0.059	
glucose metabolism	IRα		0.77	0.022	*
	IRβ		0.69	0.011	*
steroid metabolism	SHBG		0.66	0.0002	***
	HSD17B2		0.73	0.039	*
	STAR		0.64	0.060	

The analysis of liver tissue showed an up-regulation of IGF-1 (p=0.012). This agrees with the well established hypothesis, that steroid hormones increase IGF-1 gene expression and also with investigations of other groups, which showed an up-regulation for IGF-1 in feedlot cattle and rats [44; 74; 75]. Several other publications [75-77] showed in the case of an up-regulation of IGF-1 mRNA also increased concentrations of circulating IGF-1 protein levels in lambs and steers, thus it can be assumed that regulation of IGF-1 levels really occurs on transcriptional level by directly affecting the expression of the IGF-1 gene. A higher synthesis of IGF-1 in the liver, which is the main source for IGF-1, could be partially responsible for the higher serum levels [77]. Due to the para- plus endocrine action of IGF-1, it can be assumed that the growth promoting effect of anabolics on muscle tissue is mediated by circulating IGF-1 produced in liver.

Besides skeletal muscles, IGF and IGFBP are also known to have an influence on smooth muscles. IGF-1 and IGF-2, which was down-regulated (p=0.013), have been shown to stimulate the migration and proliferation of smooth muscle cells. Herein, IGF-1 is more potent in the post-natal phase than IGF-2, which exhibits its function mostly in the pre-natal phase [78; 79]. This response can be modulated by binding of IGF to IGFBP. Especially, IGFBP-2 may completely suppress the effect of IGF-1. The migration of smooth muscle cells is one of the initial events in the formation of atherosclerosis, which is the major reason for cardiovascular side effects after misuse of anabolic steroids in humans [52]. In the actual study, an up-regulation of IGF-1 and a down-regulation of IGFBP-2 (p=0.001) was recognized. This observation could be a sign of an augmented migration of smooth muscle

cells. These changes on the gene expression level and the known disturbed lipid profile [80] could cause the increased risk of atherosclerosis following abuse of anabolics.

The endocrine factor SHBG conduces to the humoral transport of steroid hormones and is mainly synthesized in the liver. Gene expression of this factor has never been examined in correlation with the action of anabolic steroids, neither in human nor in bovine, but literature describes a decreased protein level of SHBG in human users of anabolic steroids [46]. In calves, the potential of SHBG binding level to be used as biomarker for anabolic treatment has also been tested [12]. These findings go in line with our results of a down-regulation of SHBG mRNA in liver ($p=0.0002$). During the exogenous application of anabolic steroids, the organism may reduce the synthesis of SHBG on the mRNA level to restrict the transport of steroid hormones and thereby faciliate degradation of unbound steroids.

The biosynthesis of steroid hormones requires cholesterol and its biosynthesis is in part controlled by genetic expression of the enzymes HMGCoA-S, HMGCoA-R and FDFT. In our experiment, HMGCoA-S was down-regulated ($p=0.023$); but due to the fact that no regulation for HMGCoA-R or FDFT could be detected, it is difficult to make any statement concerning the influence of a treatment with TBA plus E2 on cholesterol biosynthesis.

HSD17B2 belongs to the group of oxidative steroid dehydrogenases, which direct their activity towards the inactivation of steroid hormones. HSD17B2 itself catalyzes the reaction from estradiol to estrone and testosterone to Δ4-androstendione [48]. The finding of a down-regulation of HSD17B2 ($p=0.039$) in the current study was unexpected. This enzyme should be up-regulated under the influence of exogenous hormones to accelerate the degradation of the applied substances. However, no precise information on physiological responses could be given based on gene expression results. So possibly, the inactivation of the applied hormones is regulated on another level.

The liver plays a pivotal role in glucose metabolism and homeostasis. Glucose uptake and storage as glycogen by hepatocytes is mediated by the interaction of insulin with hepatic insulin receptors [81]. In the present study, IRα ($p=0.022$) as well as IRβ ($p=0.011$) were down-regulated leading to an impaired insulin sensitivity of the liver [82; 83]. This mechanism could be responsible for the reduction of glycogen synthesis, lipogenesis and protein synthesis due to the action of anabolic steroids. As these nutrients may be less metabolized by the liver, they are kept in the blood and are available for proliferation processes in the skeletal muscles. This is consistent with the observed down-regulation of TAT ($p=0.028$) and CTSL ($p=0.009$). TAT is an enzyme that is mainly located in the liver catalyzing the first step in the degradation-pathway of the amino acid tyrosine [84; 85]. The down-regulations of the protease CTSB and the aminotransferase TAT could be responsible for an anti-catabolic

effect and for protein plus nitrogen retention. Togeteher with the up-regulation of IGF-1, these effects could be involved in muscle growth under the influence of anabolic steroids [1].
Unlike the natural androgen testosterone, which mediates its anabolic action by binding to the AR and also, after its conversion by an aromatase to estradiol, to ER (ERα and ERβ), the xenobiotic androgen TBA is able to bind to AR, GR and PR [1]. AR and GR as well as both ER subtypes have been investigated in this study. As just an up-regulation of AR ($p=0.01$) occurred, it could be assumed that the anabolic effects of the applied treatment are mainly due to TBA by its binding to AR. However, it has to be kept in mind that there are also other regulation levels of physiological function like posttransciptional modifications, which can participate in the regulation of the mentioned receptors that could not be shown to be significantly regulated in this investigation. Besides the regulation of gene transcription, steroid hormones are known to influence the stability and degradation of existing mRNA and especially to regulate the concentrations of their own receptors [86; 87]. So changes in gene expression on the transcriptional level of certain target genes could only be a part to changes in biochemical pathways.
In biomarker research, the application of valid bioinformatical and statistical tools for data processing is important to extract the required information. In most of the scopes biomarkers are searched for, generally not only one factor is used, but a variety of factors is integrated to a biomarker pattern. Therefore, especially methods for dimensionality reduction are needed to transform the high-dimensional data sets [10]. PCA has been applied as a multivariate analysis method, which can be used, if more than three components should be taken into account. PCA was produced as shown in figure 9 by plotting the expression results of regulated genes of all samples of both groups by their first and second principal component. Grey crosses represent samples of the control group, black triangles display the samples of treatment group. A distinct control group could be seen separated from the treatment group, showing that there was a multitranscriptional response to the treatment.

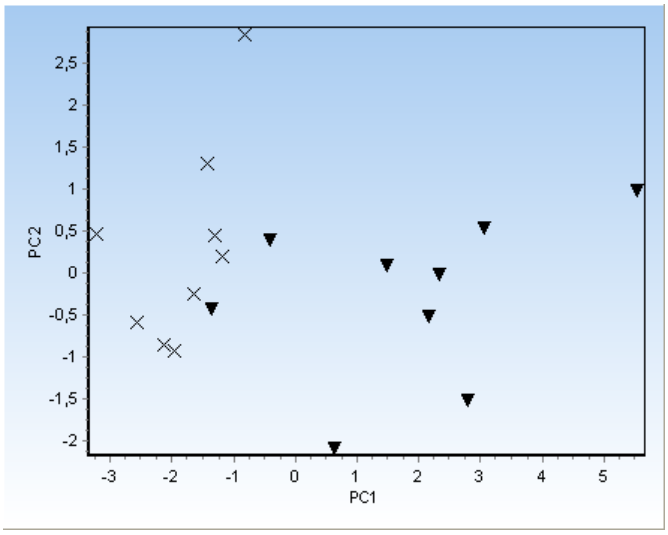

Figure 9: PCA of 15 regulated target genes from bovine liver of Nguni heifers with control animals represented by grey crosses and treated animals represented by black triangles

The PCA of all regulated genes showed that both groups arrange together and the treatment differs from the control animals although single genes are distributed within similar ranges. In addition to discussed regulations of single genes, this illustration could help to reveal the transcriptional shift in the treatment group, which could be an indication for functional changes in liver physiology following anabolic treatment. Also, this observation is a first track that it is possible to elaborate a gene expression pattern making it is possible to develop a screening method to control the misuse of anabolic hormones in cattle.

3.1.2 Influence of anabolic combinations of androgens plus estrogens on reproductive tissues in post-pubertal Nguni heifers and pre-pubertal Holstein Friesian calves

3.1.2.1 RNA integrity

RNA integrity was measured prior to gene expression analysis to prove the suitability of the sample material for RT-qPCR. In the "South Africa study", samples from uterine endometrium horn showed a mean RIN of 8.3 ± 0.49 (n=12), samples from uterine endometrium corpus showed a RIN of 7.9 ± 0.49 (n=12) and samples from ovary showed a mean RIN of 7.5 ± 0.98 (n=11).

Ovary samples from the "pour on anabolics study" showed a mean RIN of 7.7 ± 0.70 (n=19). Therefore, all samples were suitable for RT-qPCR [73].

3.1.2.2 Gene expression results from post-pubertal Nguni heifers and pre-pubertal female Holstein Friesian calves

Gene expression was measured in uterine endometrium and ovary of Nguni heifers treated with Revalor H (TBA plus E2) from the "South Africa study" and in ovary of Holstein Friesian calves treated with a hormone mix (testosterone cypionate, testosterone decanoate and estradiol benzoate), which is typically known from illegal use [7], from the "pour on anabolics study".

Gene expression results from Nguni heifers

In uterine endometrium corpus, 7 genes showed significant regulations (table 6).

Table 6: Regulated target genes in uterine endometrium corpus of Nguni heifers after steroid treatment (* = p<0.05; **= p<0.01; value 1.00 represents no expression change)

functional group	gene	x-fold regulation		p-value	significance
		↑	↓		
receptors	AR	1.71		0.012	**
angiogenesis	FGF-7	1.62		0.088	
	VEGFA	1.62		0.044	*
proliferation/ apoptosis	IGFBP-3	2.69		0.085	
	CASP3		0.82	0.005	**
	c-fos	2.73		0.028	*
	BMP-4		0.61	0.020	*
	CEBPD	2.28		0.024	*
others	Complement C7	4.42		0.022	*

Similar regulations occurred in uterine endometrium horn (table 7). Significant regulations for 8 genes could be obtained.

Table 7: Regulated genes in uterine endometrium horn of Nguni heifers after steroid treatment (* = p<0.05; **= p<0.01; value 1.00 represents no expression change)

functional group	gene	x-fold regulation		p-value	significance
		↑	↓		
receptors	AR	1.65		0.014	**
	IGF-1R		0.73	0.083	
angiogenesis	ANGPT-1		0.47	0.027	*
	MMP-2		0.65	0.057	
proliferation/ apoptosis	TNFα	5.05		0.018	*
	CASP3		0.72	0.00001	***
	FASL	1.99		0.049	*
	p53		0.72	0.076	
	BMP-4		0.64	0.041	*
protein metabolism	CTSB		0.63	0.033	*
others	Complement C7	6.92		0.001	***
	CYP19P1	1.87		0.070	

19 out of 40 investigated genes showed significant regulations in ovary (table 8). Regulations occurred in the receptors group, the angiogenesis group, the proliferation and apoptosis group, the steroid metabolism group and other factors.

Table 8: Regulated genes in ovary of Nguni heifers after steroid treatment (* = p<0.05; **= p<0.01; value 1.00 represents no expression change)

functional group	gene	x-fold regulation		p-value	significance
		↑	↓		
receptors	AR	1.67		0.028	*
	ERβ	8.19		0.010	**
	LHR		0.29	0.004	**
	FSHR	3.61		0.028	*
	ALK-6		0.56	0.083	
	FLT-1		0.64	0.050	*
	IGF-1 R	2.03		0.003	**
	PR	2.20		0.028	*
angiogenesis	VEGF 120		0.34	0.021	*
	VEGF 164		0.21	0.015	*
	FGF-2	2.06		0.038	*
	ANGPT-2	2.32		0.015	*
	MMP-2	2.46		0.007	**
	TIMP-2		0.24	0.028	*
proliferation/ apoptosis	Smad 2	1.27		0.083	
	c-jun	2.00		0.052	
	p53	1.68		0.021	*
	IGF-1	2.34		0.026	*
	BMP-2	2.64		0.005	**
steroid metabolism	S5A1		0.53	0.021	*
	HSD17B3		0.61	0.024	*
others	INHA	4.67		0.003	**

Several biochemical pathways could be shown to be differentially regulated on transcriptional level under the influence of an anabolic combination of an androgen plus an estrogen in uterine endometrium and ovary of Nguni heifers. It is generally known that anabolic steroids and especially estrogens cause a tropic response in the reproductive tract [88]. Coincidently, several factors taking place in proliferative and anti-apoptotic events were regulated in the actual study. In human ovary and uterus, AR, which is up-regulated in all tissues (p_{ovary}=0.028; $p_{uterus\ horn}$=0.014; $p_{uterus\ corpus}$=0.012), is thought to play a role in physiological proliferation and also in uncontrolled cell growth during tumorigenesis [62]. There are few data concerning AR gene expression in bovine reproductive tract, but estrogens were shown to induce AR expression in rat uterus to mediate the uterotropic effect [89; 90]. Our data

suggest that AR is targeted by anabolic steroids in a similar way in bovine uterus and ovary triggering cell growth.

Concordantly, the transcription factors CEBPD (p=0.024) and c-fos (p=0.028), which were shown to be implicated in cell turnover under the influence of steroid hormones [56; 58; 62; 91], were up-regulated. Additionally, the up-regulated TNFα (p=0.018) is known to be involved in proliferation and differentiation of in the uterus [92]. This rise in expression of several proliferative factors could be linked with a higher proliferation rate of the organ.

While cell growth was induced, a parallel inhibition of apoptotic and tissue degrading factors occurred. BMP-2 and BMP-4 are known to cause apoptosis in many different target cells and has been proven to be implicated in tissue remodeling of the cyclic uterus [93; 94]. Also, the protease CTSB plays a role in the degradation of extracellular matrix and the catabolism of intracellular proteins and is therefore physiologically involved in the tissue remodeling of the cyclic uterus [65]. The down-regulations of BMP-2 (p=0.005), BMP-4 ($p_{uterus\ corpus}$=0.020; $p_{uterus\ horn}$=0.041) and CTSB (p=0.033) propose an inhibition of tissue breakdown and thereby an increase in organ weight in the treated animals. CASP3 is one of the key mediators in apoptosis [64]. Its down-regulation ($p_{uterus\ corpus}$=0.053; $p_{uterus\ horn}$=0.00001) in uterus in the actual study could give a hint for the inhibition of apoptosis under the influence of anabolic treatment. In ovary, an up-regulation of the p53 (p=0.021) tumour surpressor gene could be detected. p53 is known to be an inhibitor of caspase activity [64; 95]. Possibly caspase-dependent apoptosis in ovary is inhibited by the action of p53. However, it has to be stated that apoptosis is a complex physiological process, which includes a variety of factors. So, no proven statement could be given from gene expression measurement of one inducing and one inhibiting factor.

Concerning proliferation in uterus, the expression of factors associated with IGF show another situation. It is generally known that IGF-1 is one of the major growth factors implicated in the proliferation of the uterus [96; 97]. The actual study showed a trend towards down-regulation of IGF-1 R (p=0.083) indicating a lower responsiveness of the organ. Also, a trend for up-regulation of the inhibitory regulator IGFBP-3 (p=0.085) could be observed. These regulations might demonstrate a protective adaption mechanism of the organism to prevent the action of the plentiful IGF-1 originating from liver [113].

Contrarily, an up-regulation of IGF-1 (p=0.026) and IGF-1 R (p=0.003) occurred in ovary. Up-regulation of IGF-1 R indicates a higher IGF-1 sensitivity. In this study, IGF-1 gene expression has been shown to be elevated in the ovary itself and also in liver [113]. Due to the autocrine and paracrine action of IGF-1 a strong proliferative response to anabolic steroids in the ovary can be expected.

A possible adaption mechanism of the organism to the anabolic treatment might also be observed in the regulation of factors implicated in ovarian steroid synthesis. HSD17B3 (p=0.024) and S5A1 (p=0.021), whose enzymatic reactions are directed towards the generation of active steroid hormones, were down-regulated. This suggested an inhibition of endogenous hormone synthesis and formation of active hormones as a negative feedback response to exogenous application of anabolics. The physiological synthesis and secretion of endogenous steroid hormones from the gonades is stimulated by the release of FSH and LH from the pituitary. The down-regulation of LHR (p=0.004) might reflect the lower responsiveness towards the LH stimulus amplifying the negative feedback. The up-regulated factor INHA (p=0.003) is known to be a suppressor of FSH secretion and could therefore be part of the negative feedback control mechanisms of hormone levels [68]. These data showed clearly that the regulatory negative feedback mechanisms were visible on gene expression level.

The female reproductive tract is one of the few adult organs showing angiogenesis independently of pathophysiology [59]. This process of vascular growth and decline is regulated by the action of reproductive hormones and could be disturbed by exogenous steroid hormones. In the ovary, VEGF and the VEGF-receptor FLT-1 are mainly implicated in the formation of new capillary networks during follicular maturation. The expression of VEGF is mainly stimulated by LH secreted from the pituitary [59]. The androgenic drug TBA applied to Nguni heifers in the actual study has a strong anti-gonadotropic ability leading to a decreased LH release. This known effect goes in line with the observed down-regulation of LHR in the ovary and thereby a lower responsiveness of this organ towards LH. The lack of the LH stimulus may be responsible for the down-regulation of the VEGF isoforms ($p_{VEGF\ 120}$=0.021; $p_{VEGF\ 164}$=0.015) and their receptor FLT-1 (p=0.050). The resulting inhibition of follicular maturation could partly be responsible for reproductive perturbations like the delayed onset of puberty shown in pre-pubertal animals or the predisposition for non-ovulatory estrus observed in mature cows under the influence of steroidal growth promoters [98]. The changes in gene expression observed in the actual study could also be accounted to mimic the state of regression of the corpus luteum (CL) characterized by a down-regulation of VEGF and an up-regulation of FGF-2 (p=0.038) and ANGPT-2 (p=0.015) [99]. Thereby, these results could possibly indicate a degrading effect of anabolic steroids on the CL and follicle. In general, there might be an inhibitory effect on angiogenic processes under the influence of anabolic steroid hormones. However, it is hardly possible to give a reliable statement on the physiological networks as little is known about the cyclic state of the

experimental animals and also about the sample material, as samples have been taken as total-ovary and have not been differentiated for cell types.

PCA for uterine endometrium (figure 10A+B) and ovary (figure 10C) of Nguni heifers was produced by plotting gene expression results of all regulated target genes from all samples of both groups by their first and second principal component. Additionally, all regulated target genes from all three organs were summarized in one PCA representing the whole reproductive tract (uterine endometrium, ovary) (figure 10D).

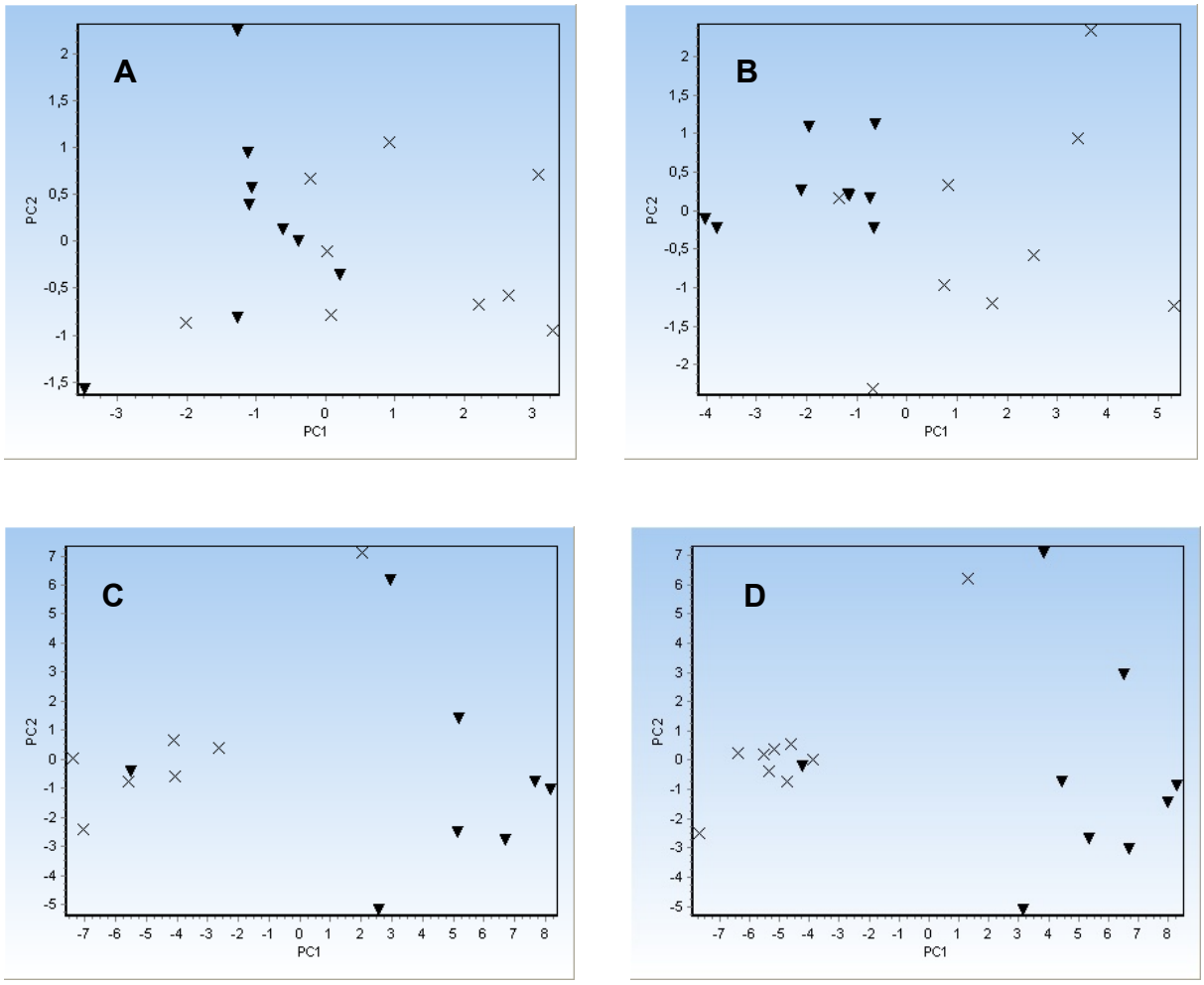

Figure 10: PCA of regulated target genes in uterine endometrium corpus (9 genes) (A) and horn (12 genes) (B), in ovary (23 genes) (C) and the whole reproductive tract including uterine endometrium plus ovary (44 genes) (D) of Nguni heifers with control animals represented by grey crosses and treated animals represented by black triangles

In all single tissues, a distinct separation between the control group and the treatment group could be observed showing that there was a multitranscriptional response to the treatment.

Also, a clear assignment could be seen for the combination of all tissues. The best separations were achieved for ovary and the composition of all three target organs making these possibilities to the most promising regarding biomarker research.

Gene expression results of Holstein Friesian calves

In ovary of pre-pubertal Holstein Friesian calves, 23 of 42 measured target genes showed significant regulations. Unexpectedly, some regulations could already be detected in the carrier control group (table 9).

Table 9: Regulated genes in ovary of Holstein Friesian calves after steroid treatment (value 1.00 represents no expression change; n.s. represents non-significant results)

fuctional group	gene	Carrier Control		1x		3x	
		x-fold	p-value	x-fold	p-value	x-fold	p-value
receptors	AR		n.s.	0.71	0.073		n.s.
	ERα		n.s.		n.s.	1.89	0.098
	PR	0.37	0.028		n.s.	1.93	0.082
	FLK-1		n.s.	3.75	0.069		n.s.
	LHR	1.78	0.032	2.06	0.014		n.s.
	ALK-6		n.s.	3.44	0.013		n.s.
angiogenesis	VEGF 120	0.78	0.054		n.s.		n.s.
	VEGF 164	0.70	0.030		n.s.		n.s.
	MMP-2		n.s.		n.s.	3.90	0.016
	MMP-23B		n.s.	1.63	0.026	1.67	0.043
	ANGPT-2		n.s.	1.90	0.090		n.s.
	TIMP-2		n.s.	3.78	0.008		n.s.
	FGF-1		n.s.	2.25	0.008		n.s.
proliferation	BMP-2		n.s.		n.s.	2.87	0.063
	IGFBP-2		n.s.	2.73	0.012		n.s.
	BMP-15		n.s.		n.s.	4.81	0.058
	c-kit		n.s.	1.93	0.055		n.s.
	RB-1		n.s.	1.72	0.095		n.s.
steroid metabolism	CYP19A1	0.26	0.025		n.s.		n.s.
	S5A1		n.s.	1.96	0.030		n.s.
	HSD3B1		n.s.	4.67	0.097		n.s.
	HSD17B3		n.s.	1.47	0.034		n.s.
	HSD17B8		n.s.	2.01	0.022		n.s.
	HSD17B11		n.s.	2.06	0.064	1.91	0.031
	STAR		n.s.	2.49	0.053		n.s.

Considering the “pour on anabolics study”, most of the results in ovary were unexpected and are hardly to discuss. The proliferative trend observed in Nguni heifers could not be confirmed in pre-pubertal calves. Although several factors, which are implicated in proliferative events like c-kit (p=0.055) or RB-1 (p=0.095) [62; 68] showed a trend for up-

regulation, none of these could reach significance. In addition, the important growth factor IGF-1 showed no up-regulation as it does in heifers. On this basis, no distinct statement could be made on a proliferative trend in ovary of calves under the application of anabolic steroids.

Contrarily to heifers, in pre-pubertal calves S5A1 (p=0.030), HSD17B3 (p=0.034) and HSD17B8 (p=0.022) were up-regulated. HSD17B8 is responsible for the degradation of E2 into the lower active estrone [48], therefore this up-regulation could be estimated as an induction of decomposition of the applied exogenous hormones. Also, in STAR, which is responsible for the transport of cholesterol from the outer to the inner mitochondrial membrane catalyzing the first step in hormone generation, a trend for down-regulation was observed (p=0.053) suggesting an inhibition of steroid synthesis. Whereas, the enzymes HSD17B3 and S5A1 were induced, not reflecting the possible protective mechanism, which was hypothesized in Nguni heifers. This regulation pattern gave no clear mark of a trend in the ovary of pre-pubertal calves concerning steroid hormone synthesis.

Concerning angiogenesis, in pre-pubertal calves, several pro-angiogenic factors like MMP-2 (p=0.016), MMP-23B (p_{1x}=0.026; p_{3x}=0.043) and FGF-1 (p=0.008), as well as the mediating receptors FLK-1 (p=0.069) and ALK-6 (p=0.013) were up-regulated. This would suggest an increase in angiogenic events after the application of exogenous hormones. However, also the anti-angiogenic inhibitor TIMP-2 (p=0.008) and the blood vessel degrading factor ANGPT-2 (p=0.090) were up-regulated prohibiting an explicit conclusion on angiogenic events.

In general, even though the same genes were regulated compared to the ovary of Nguni heifers, the direction of the regulation differed, which was obvious especially in receptors (AR, PR, LHR), but also for steroidogenic enzymes (HSD17B3, S5A1). This could possibly be explained by the different hormonal status of calves and heifers. Accessorily, the reproductive tract of the calves might show no responsivity to the exogenous application of steroid hormones due to the rudimentary developmental stage. A similar hypothesis has been introduced by Cacciatore et al. [12], who observed no effect of hormone administration on different steroid target genes in pre-pubertal animals. Also, different anabolic preparations as well as different application modes (permanent implants versus pour on treatment) may cause different transcriptional responses in the experimental animals.

Especially, the route of application may be a reason for the unexpected results as rare valid data exist concerning the pharmacokinetics of steroid esters after pour on treatment. In the course of this study, co-workers from RIKILT (Institute of Food Safety, Wageningen, The Netherlands) investigated the occurrence of the applied steroid hormone esters in hair and

plasma. Stolker et al. were able to find the applied steroid esters in hair [7]. However, concentrations were under the detection limit after 5-7 weeks (1x treatment) and 9-11 weeks (3x treatment), respectively. Furthermore, no free steroids released from the applied esters could be detected in plasma. These results indicate that the administered steroid esters compass hair via sweat or sebum excretion at the surface of the skin and reach the blood stream only in negligible amounts. Also, the short duration of the drug effect could be proven. At the time point of slaughter, the applied substances have already been eliminated from the organism, which could possibly be a reason for the absence of specific gene expression changes [7]. In addition, significant regulations already occurred in the carrier control group for VEGFs, PR, LHR and CYP19A1. This group was integrated in the trial to estimate the possible effect of the applied carrier substances. Due to these regulations, the influence of the carriers and the real anabolic treatment can hardly be differentiated. In fact, it has to be discussed, if the regulations in the treatment groups are really due to the anabolic treatment or arise from inter individual variations from animals in a high developmental stage.
In the PCA of ovarian tissue for pre-pubertal calves, all significantly regulated genes in all four experimental groups were plotted (figure 11).

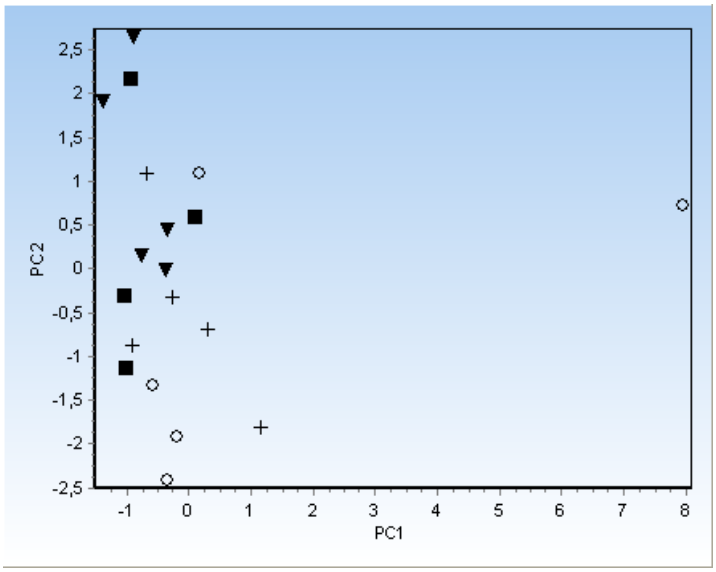

Figure 11: PCA of 23 regulations measured in ovary of Holstein Friesian calves
(○= control group, + = carrier control group, ▼= 1x treated group, ■ = 3x treated group)

Obviously, no separation could be seen between the two control and the two treatment groups making it debatable, if there was a response to the anabolic treatment on transcriptional level. Thus, the pattern of genes, the immature reproductive tract or the age

class of animals seems to be less suitable for surveillance of anabolic treatment using transcriptomics. Independently of the gene expression results, an anabolic effect of the treatment was visible on the phenotype level by comparing weight gain and carcass weight (unpublished in-house data). Herein, the highest differences could be observed between the control animals and animals from the 3x treatment group on days 28, 63 and 91 after beginning of the treatment. Also the carcass weight at slaughter was significantly higher in the 3x treated group. No differences could be detected between the two treatment groups. These data show that an anabolic effect using pour on application becoming manifest in significantly increased weight gain might not be visible at the gene expression level and might therefore remain undetected, when using transcriptomics for surveillance.

For biomarker research, these results indicate that it might be necessary to establish different biomarkers for specific treatment regimes and different age classes of animals.

3.2 miRNA studies

3.2.1 Validation of miRNA analysis

3.2.1.1 miRNA extraction

Two kits (*mir*Vana, Applied Biosystems; miRNeasy Mini Kit, Qiagen) have been tested to validate a suitable miRNA extraction method. For testing, randomly picked samples from uterine endometrium and ovary of Nguni heifers from the South Africa study were used (n=6). In the first instance, for both kits the “two-tube-extraction” gaining two separated RNA fractions was applied. Similar results could be obtained for RNA yield, with a mean sample concentration of 93.64 ± 55.44 pg/µL for small RNA and 962.32 ± 357.15 pg/µL for long RNA in the *mir*Vana kit and a mean sample concentration of 77.71 ± 49.05 pg/µL for small RNA and 989.40 ± 719.67 pg/µL for long RNA in the miRNeasy Mini Kit. The high standard deviations are due to the samples, which are different biological samples and not replicates. Extracts from both kits were then analyzed using the Small RNA Assay (figure 12A-C) and the Eukaryote Total RNA Nano Assay (figure 13A-C) on the Agilent 2100 Bioanalyzer.

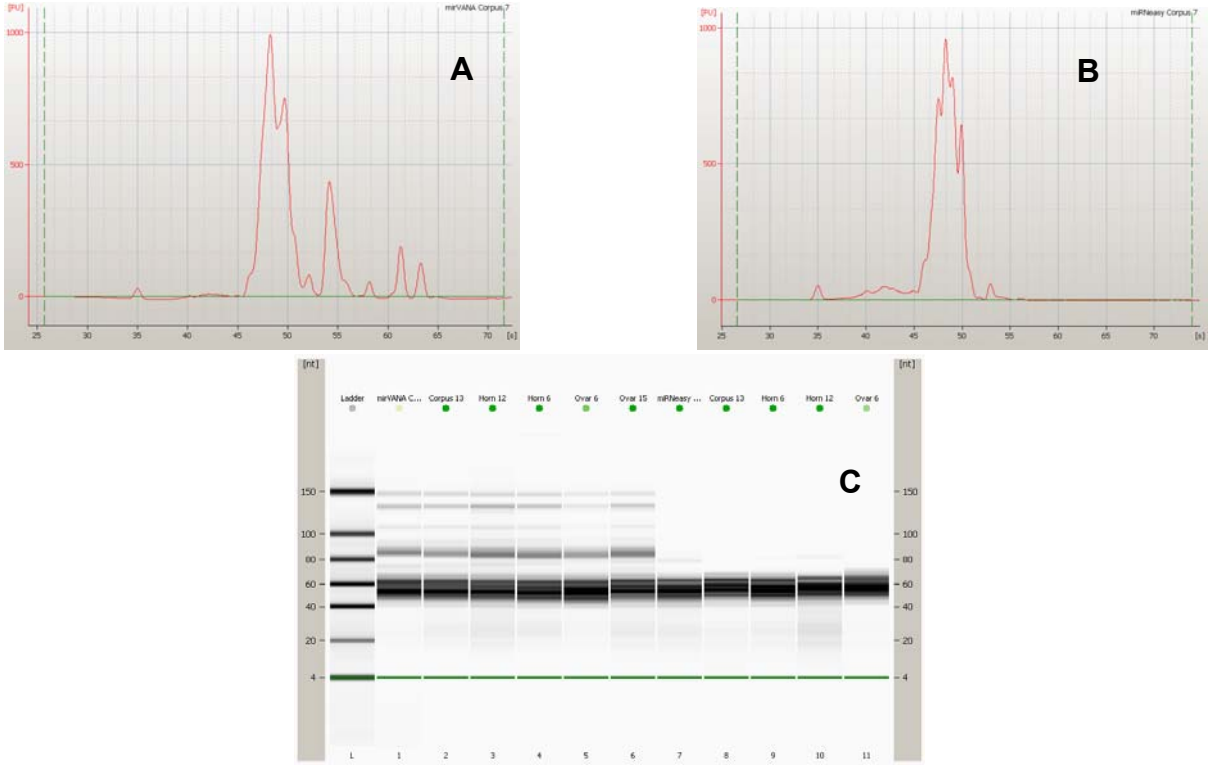

Figure 12: RNA extracts from the *mirVana* (A) and the miRNeasy Mini Kit (B) analyzed on the Small RNA Assay, the gel-like image (C) and the electropherogram clearly indicate unspecific peaks and bands in the extracts obtained from the *mirVana* Kit

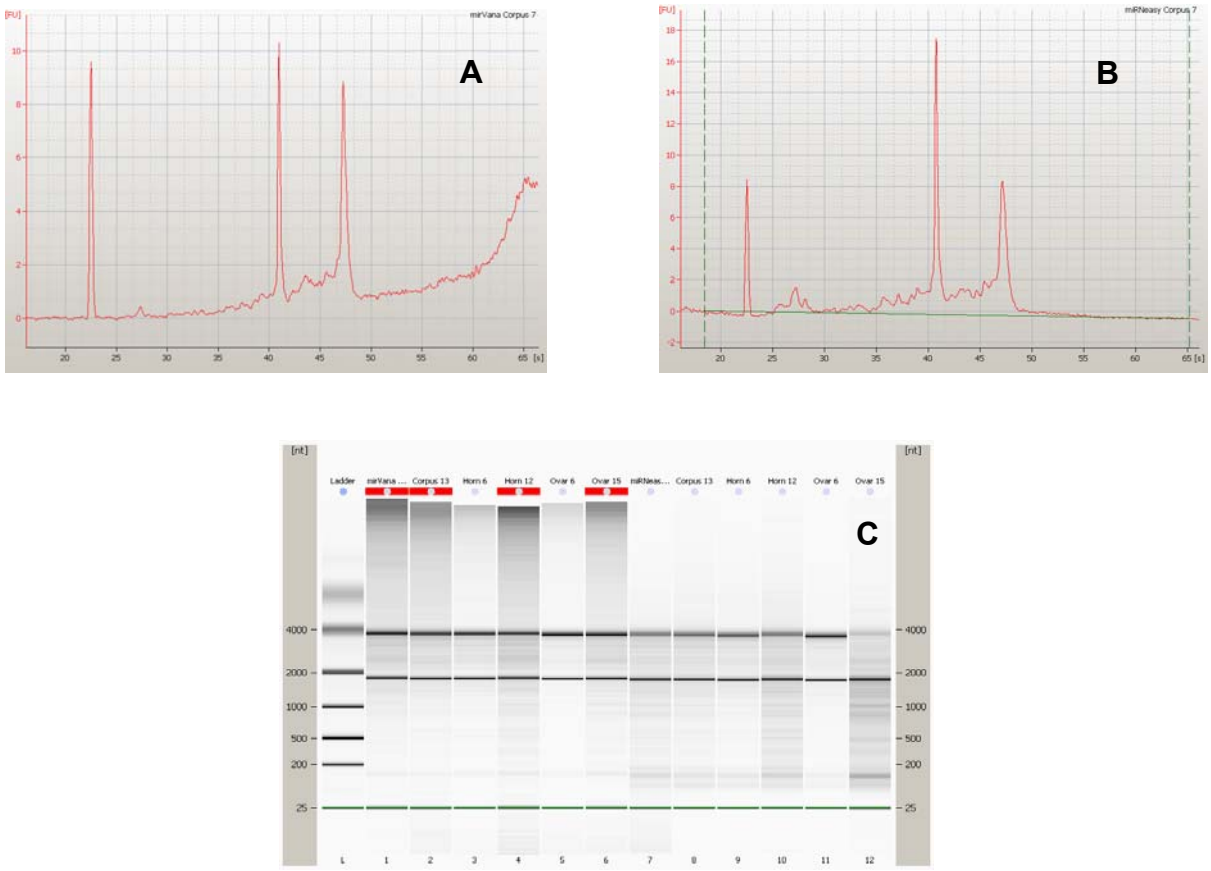

Figure 13: RNA extracts from the *mirVana* (A) and the miRNeasy Mini Kit (B) analyzed on the Eukaryote Total RNA Nano Assay, the gel-like image (C) and the electropherogramm clearly indicate unspecific peaks and bands in the extracts obtained from the *mirVana* kit

The electropherograms as well as the gel-like image showed better purification of nucleic acids as well for small RNAs as for long RNAs in extracts from the miRNeasy Mini Kit.

The Small RNA Assay as well as the Eukaryote Total RNA Nano Assay showed unspecific peaks in the electropherogram and unspecific bands in the gel-like image of extracts, which were obtained from the *mir*Vana Kit. This can only be explained by unspecific residues from the biological sample, which were not removed by the *mir*Vana Kit.

No investigation of RNA integrity was possible for extracts from the *mir*Vana kit also due to residual artifacts, whereas the miRNeasy Mini kit gave good results for RNA integrity with a mean RIN of 7.68 ± 1.11. All chips with questionable results have been repeated to exclude technical failure. Due to these observations, the miRNeasy Mini kit has been chosen for further validation.

Continuing, the suitability of the “one-tube-” versus the “two-tube-extraction” has been tested. Three samples (muscle tissue) have been extracted in duplicate with both extraction

methods. Examplary, results gained from one sample are shown in figure 14. Similar results were obtained for the remaining samples. Extracted RNA fractions have been analyzed in duplicate using the Eukaryote Total RNA Nano Kit on 2100 Bioanalyzer (figure 14A+B). The small RNA fraction from the “two-tube-extraction” has also been analyzed in the Eukaryote Total RNA Nano Assay (figure 14C).

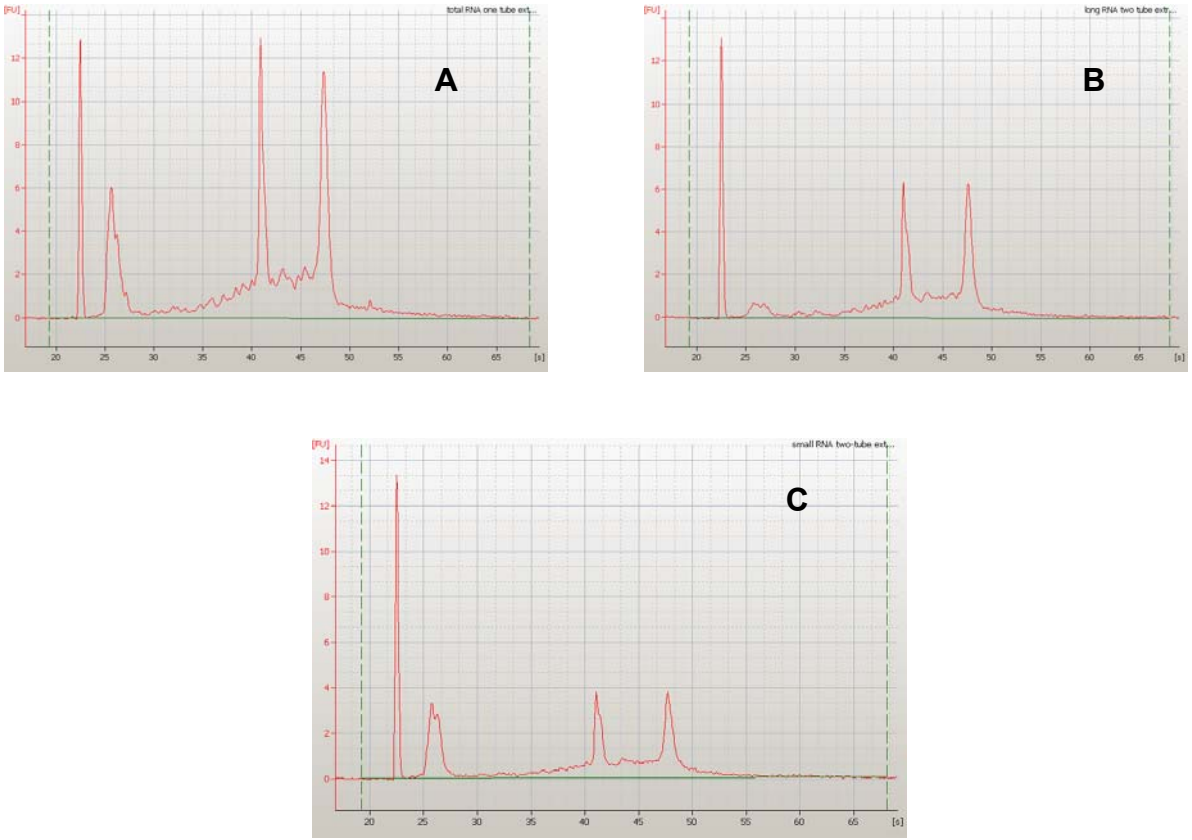

Figure 14: RNA extracts of the same sample with the “one-tube” and the “two-tube-extraction-method”, respectively; total RNA fraction (A), long RNA fraction (B) and small RNA fraction (C) were analyzed with the Eukaryote Total RNA Nano Assay

Both methods generate extracts with good RNA quality with a mean RIN of 8.1 ± 0.21 for the total RNA fraction from “one-tube-extraction” and 8.4 ± 0.25 for the long RNA fraction of the “two-tube-extraction” (n=12). It was expected that the calculated RNA integrity might be lower in the “one-tube-extraction”. In this method, the small RNA fragments, which include functional small RNAs as well as degraded RNA fragments, have not been separated from the long RNA fraction. This small RNA fraction appears in the front region of the electropherogram and is accounted as a signal of degradation by the software biasing the

RIN calculation. Due to that, the RNA integrity of extracts from the “one-tube-extraction” is estimated to be lower.
When measuring the small RNA fraction from “two-tube-extraction” on the Eukaryote Total RNA Nano chip, distinct peaks for 18S and 28S RNA were visible, even indicating an RIN of 8.0 ± 0.45 (n=10). This result showed that there is no distinct separation between the long RNA and the small RNA fraction applying the “two-tube-extraction” making it impossible to obtain a valid concentration measurement as both fractions demonstrate a mixture of all RNA species. So, the “one-tube-extraction” has been chosen for further analysis and total RNA concentration was used to determine the template quantity for further analysis.

3.2.1.2 Verification of the Small RNA assay on the Agilent 2100 Bioanalyzer

For the verification of the Small RNA Assay concerning specificity and reproducibility, five different tissues (kidney, liver, heart, intestine and muscle) and WBC, which were extracted in six replicates (n=6) with the “one-tube-extraction”, were screened for their miRNA concentration. Chips were run two times each on two different days, so four times overall. As numerical results, the miRNA/small RNA ratio [%] and the miRNA concentration [pg/µL] were determined. Summarizing all tissues, in the calculation of the miRNA concentration, the Small RNA Assay showed a mean inter-chip CV of 28.47 ± 10.47% and an intra-chip CV of 18.16 ± 8.12%. For the calculation of the miRNA/small RNA ratio a mean inter-chip CV of 21.45 ± 9.42% and a mean intra-chip CV of 16.38 ± 8.76% could be observed. Obviously, the Small RNA Assay shows no valid reproducibility and thereby no reliable quantification of small RNA. These results give another evidence for the better suitability of the “one-tube-extraction” to determine the miRNA amount for further analysis from the concentration of total RNA, which can reliably be measured photometrically.
In the screening of the different tissues for their miRNA concentration an interesting observation was made. Those samples, with a high degree in degradation represented by a lower RIN, seemed to have the highest concentration of miRNA (table 10).

Table 10: Mean RNA integrity represented by the RIN, absolute amount of miRNA [pg/µL] and miRNA/small RNA ratio [%] (n=6) of five different tissues and WBC screened in the course of validation of the Small RNA Assay

	Heart	Muscle	Intestine	Kidney	WBC	Liver
Ø RIN	8.38 ± 0.32	8.35 ± 0.28	8.15 ± 0.40	5.51 ± 0.47	9.53 ± 0.25	7.98 ± 0.25
Ø miRNA/smallRNA ratio [%]	7.50 ± 2.52	4.45 ± 1.00	12.35 ± 1.42	21.15 ± 3.00	5.86 ± 1.91	8.00 ± 1.45
Ø miRNA concentration [pg/µl]	885.455 ± 224.54	850.67 ± 291.23	2347.81 ± 394.69	6751.355 ± 2231.9	711.19375 ± 241.82	1522.405 ± 623.49

The question arose, if degraded RNA fragments might reach the analytical range of the Small RNA Assay and may disturb the analysis.
Therefore, the influence of total RNA integrity on small RNA quantification and miRNA expression analysis should be investigated.

3.2.1.3 Impact of total RNA integrity on small RNA quantification and gene expression analysis

The influence of overall RNA degradation on small RNA quantification and gene expression analysis was investigated in three different tissues (WBC, muscle, liver).

RNA degradation

RNA degradation via UV light was successful in all tissues and an integrity gradient could be created (exemplary the results for WBC are shown in table 11 and figure 15). As RNA quality consists of RNA integrity and RNA purity, also the $OD_{260/280}$ ratio and the $OD_{260/230}$ ratio have been checked photometrically to ensure good RNA purity. This examination showed satisfying and constant RNA purity for all degradation steps with a mean $OD_{260/280}$ of 2.033 ± 0.027 and a mean $OD_{260/230}$ of 1.925 ± 0.14 (n=28) indicating that the different degradation levels are solely caused by a shift in RNA integrity.

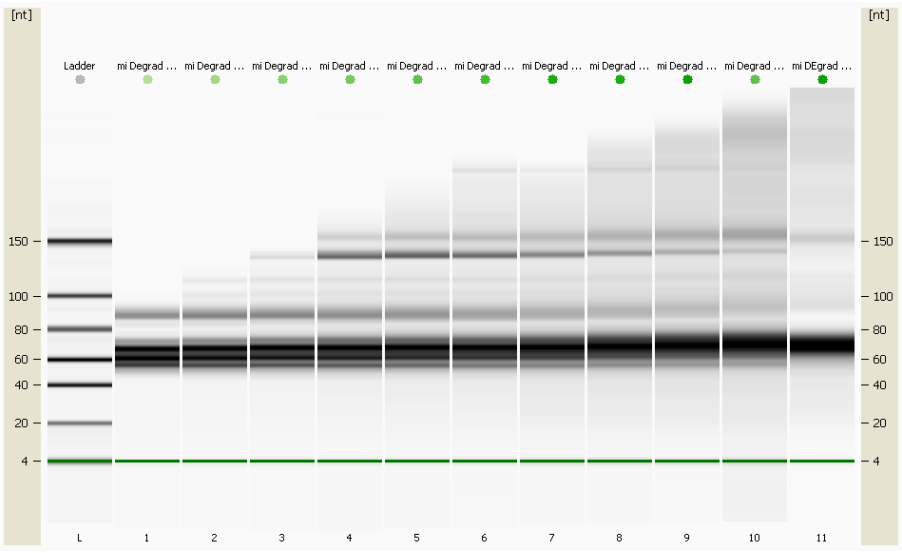

Figure 15: Electronic gel-like image on the Small RNA Assay from the results of UV-based degradation of WBC

miRNA quantification

miRNA quantification using the Small RNA Assay showed a clear relation between the miRNA amount and the state of degradation for all tissues. With ongoing RNA degradation a highly significant rise ($p<0.001$) in miRNA/small RNA ratio appeared for all tissues (figure 16).

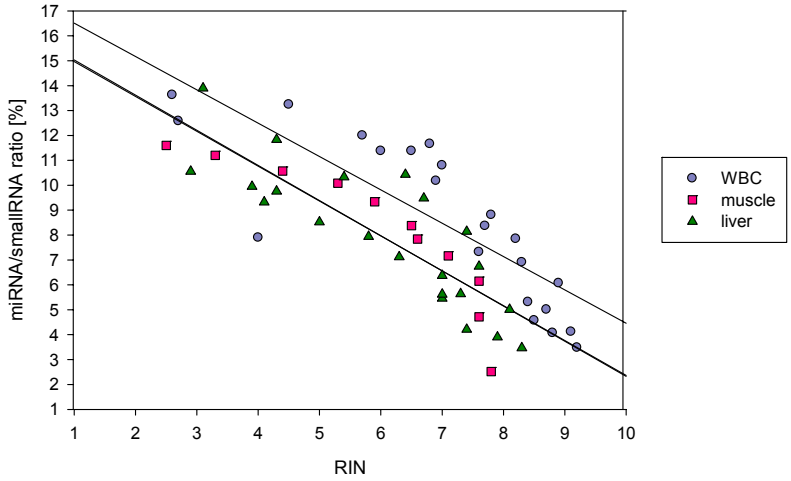

Figure 16: Highly significant correlation between RIN and miRNA/smallRNA ratio [%] for liver, muscle and WBC with $p<0.001$ for all subsets (regression lines for liver and muscle are overlapping)

In liver and WBC, also a significant increase ($p<0.001$) in the miRNA concentration occurred. An increase could also be shown for muscle tissue, but the rise was not statistically significant.

In WBC, the miRNA/small RNA ratio ascended from 3.80 ± 0.45% to 13.11 ± 0.74% showing a clear increase due to the formation of short RNA fragments during RNA degradation. Similar results were acquired for liver and muscle. For muscle, an increase from 2.19 ± 0.47% to 11.08 ± 0.74% and for liver, an increase from 3.69 ± 0.30% to 12.23 ± 2.36% was shown. A comparable relationship was obtained for the correlation between RIN and miRNA concentration ($p < 0.001$). The miRNA concentration in liver rose from 999.92 ± 43.63 pg/µL to 2697.35 ± 616.21 pg/µL, in muscle from 814.49 ± 163.73 pg/µL to 2902.54 ± 306.89 pg/µL and in WBC from 592.54 ± 17.76 pg/µL to 2053.08 ± 47.11 pg/µL. An exemplary summary of the miRNA quantification results for WBC on the 2100 Bioanalyzer is stated in table 11.

Table 11: RIN values and miRNA quantification data from the Bioanalyzer exemplary for WBC

good quality RNA [%]	mean RIN	SD RIN	mean miRNA/small RNA ratio [%]	SD ratio [%]	mean miRNA concentration [pg/µl]	SD miRNA concentration [pg/µl]
100	9.15	0.07	3.80	0.45	592.54	17.76
90	8.75	0.07	4.54	0.66	765.47	69.21
80	8.65	0.35	5.69	0.54	1031.72	222.49
70	8.40	0.14	5.74	1.65	1141.24	129.56
60	7.90	0.42	7.59	0.37	1455.04	380.61
50	7.75	0.07	8.59	0.31	1643.46	563.81
40	6.95	0.07	10.49	0.44	1586.82	475.17
30	6.65	0.21	11.52	0.20	1897.65	773.89
20	5.85	0.21	11.69	0.44	1805.73	339.21
10	4.25	0.35	10.57	3.78	1623.96	808.92
0	2.65	0.07	13.11	0.74	2053.08	47.11

As expected, a significant rise in miRNA amount occured with ongoing RNA degradation. This is caused by the formation of small RNA fragments by degradation of longer RNAs. Due to their length, these small fragments could reach the analytical range of the Small RNA Assay and therefore lead to an overestimation of the miRNA amount. Hence, the concentration measurement using the 2100 Bioanalyzer is just reliable for RNA samples with good RNA quality. In consequence, it is recommended to consider the miRNA not as an isolated solitaire fraction, but always in combination with the mRNA and total RNA. Also, the definition of the miRNA fraction on the Small RNA Assay is questionable. Herein, all fragments with a length of 15-40 nt are defined as miRNAs, although in literature miRNAs are considered to have a length of 18-25 nt [100]. Thereby, it may be expected that even in samples with a low degree of fragmentation undefined RNA fragments are accounted as

miRNAs and the miRNA amount may be overestimated even in samples with good RNA integrity. These results show clearly that it is hardly possible to quantify exactly the real amount of miRNA in a biological sample with existing methods. Coming technical innovations may give the possibility to solve these complex problems.

Validation of the miRNA qPCR assay

Gene expression analysis of bovine miRNAs has been done using a commercial kit in combination with human primer assays (miScript System, Qiagen). After cDNA synthesis and adding 40 µL of RNAse free water to a final volume of 60 µL, a 1:10 dilution series with 7 steps was created to test the linearity of the assay (figure 17). In this assay, let-7a was measured as target gene showing 100% sequence homology between human and bovine.

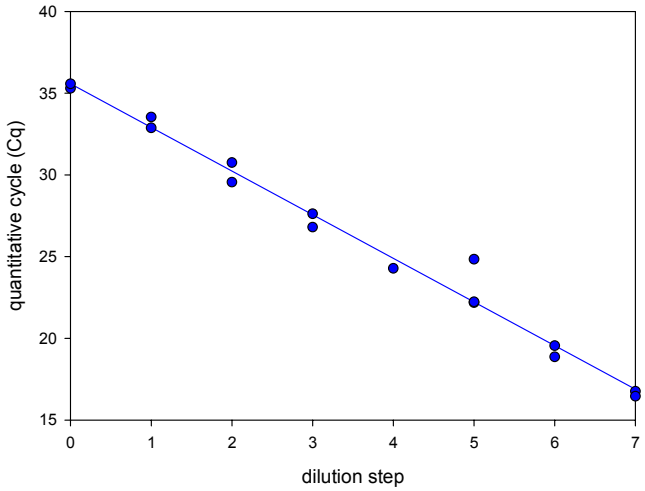

Figure 17: Linearity of the miRNA qPCR assay

Resulting gene expression data were linearly regressed and showed a highly significant ($p<0.001$) linearity of the assay (slope=2.67, $r^2=0.98$) indicating suitable performance of the qPCR even in the low template range.

Melting curve analysis showed one distinct peak demonstrating the generation of one specific product. In combination with the sequencing of the amplification products (see below), these results prove product specificity of the qPCR.

Sequencing of miRNA PCR products

As human miRNA primers were used for bovine miRNA amplification, it should be controlled if specific products were amplified in qPCR, even if sequences show no 100% homology between human and bovine.

As expected, sequencing of amplification products from miRNA expression analysis showed specific products for amplified bovine sequences, which were 100% homologous to the human sequence. In the case of 3´abberation in the bovine sequence compared to the human sequence, results for sequencing were unclear. If the bovine sequence showed an additional base at the 3´end, the human primer assay did not amplify the specific product and different base types were integrated in the sequence. If the bovine sequence showed an absent base at the 3´end compared to the human sequence, the human amplification product was synthesized. However, in this case it can be estimated that the bovine miRNA is targeted by the human primer and the additional base is added during amplification. So, it could be shown that human primer assays can be used riskless for bovine sequences with 100% homology to the respective human sequence. In case of aberrations, gene expression results should be checked carefully and should be validated by sequencing.

From another point of view, these results indicate the specific performance of the primer assays. Herein, human primer assays are able to distinguish between different members of miRNA families (e.g. let-7 family), which mainly differ just in one nucleotide aberration at the 3´end.

Results of gene expression analysis

Gene expression of mRNA and miRNA was measured using RT-qPCR. As expected, a distinct and highly significant, negative correlation between RIN and Cq could be shown for mRNA in all tissues and for all quantified genes (r^2_{mean} = 0.837; $p<0.001$) with a mean slope of the regression line of 1.578. Exemplary, the gene expression results for WBC are demonstrated in figure 18. Analog results were obtained for muscle and liver tissue.

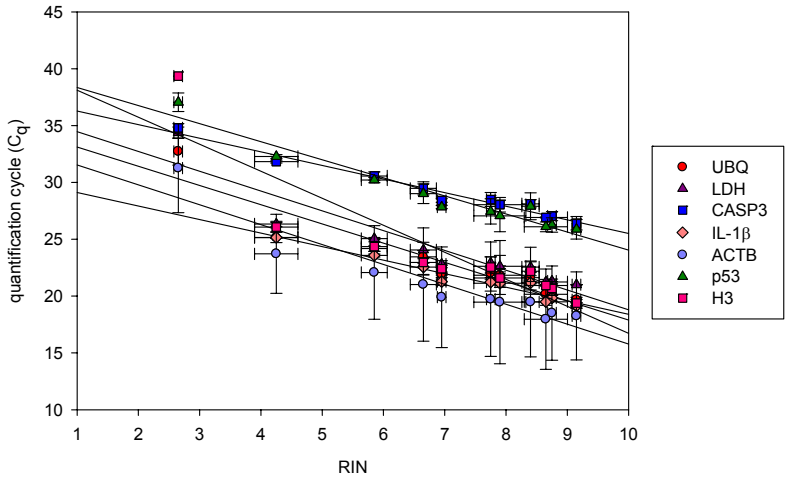

Figure 18: mRNA expression data obtained from WBC showing a highly significant correlation between RIN and Cq value with p<0.001 for all subsets

Comparable to mRNA expression, a highly significant correlation between the RIN and the Cq value (r^2_{mean} = 0.835; p<0.001) could also be observed for miRNA expression with a mean slope of the regression line of 0.784. Expression results for WBC are shown in figure 19.

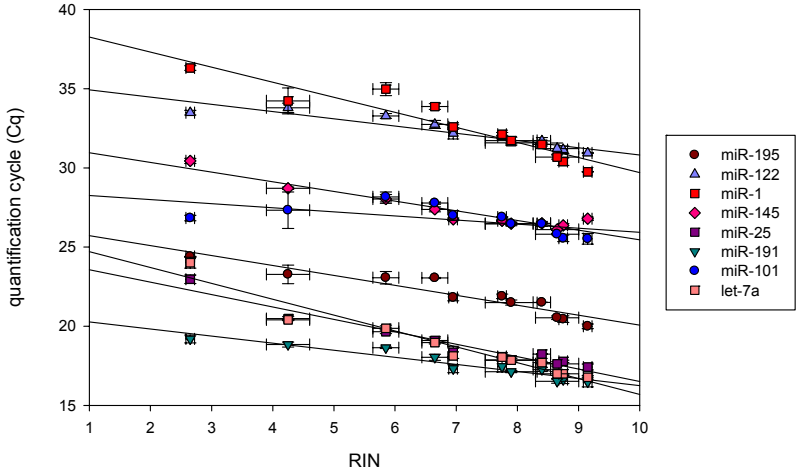

Figure 19: miRNA expression data obtained from WBC showing a highly significant correlation between RIN and Cq value with p<0.001 for all subsets

For gene expression analysis, mRNA as well as miRNA profiles have been investigated in this study to get a whole view over gene expression and to verify former results. A highly significant, negative correlation between the RIN and the Cq value for mRNA could be observed. Therefore, the results of earlier studies of Fleige and coworkers could be clearly

confirmed [34; 35]. Similar to mRNA, there was also a highly significant, negative correlation between the RIN and the Cq value for miRNA. From the lower slope of the regression line (1.578 vs. 0.784), it could be inferred that the compromising effect is less pronounced in comparison to mRNA. Due to their length miRNAs seem to be more stable and exhibit less recognition sites for nucleases. We conclude that miRNAs might be less affected by the overall degradation of total RNA compared to longer mRNAs.

Normalization of expression data

mRNA expression data have been normalized using a reference gene index consisting of the geometric mean expression of two suitable RG, which were determined for every tissue by GenEx software. Optimal number of RG was selected using pairwise variation analysis integrated in geNorm algorithm implented in GenEx [101]. For WBC, Actinβ (ACTB) and LDHA; for muscle tissue, UBQ and ACTB and for liver tissue, ACTB and CASP3 were used for calculation of an RG index. Normalized expression data were linearly regressed with the RIN. For almost all data, no statistically significant correlation between the RIN and the ∆Cq value could be shown. Significant correlations were consistent after normalization in muscle for CASP3 and Interleukin 1β (IL-1β), in liver for UBQ and IL-1β and in WBC for CASP3 and H3. For these genes, the correlation was no more significant, when eliminating the results from degradation step 10 and 11 (10 % and 0% good quality RNA), clearly showing that samples with a very low RIN are not suitable for qPCR analysis.

Two strategies were applied for normalization of miRNA expression data. Similar to the determination of RG for mRNA, suitable RG for miRNA were detected using GenEx software. For WBC, miR-122 and miR-191; for muscle and liver tissue, miR-122 and let-7a were used for calculation of an RG index [101].

As second strategy, normalization using the geometric mean expression value of all measured miRNAs was applied [102]. Examples for both normalization methods obtained in WBC are shown in figure 20 A+B.

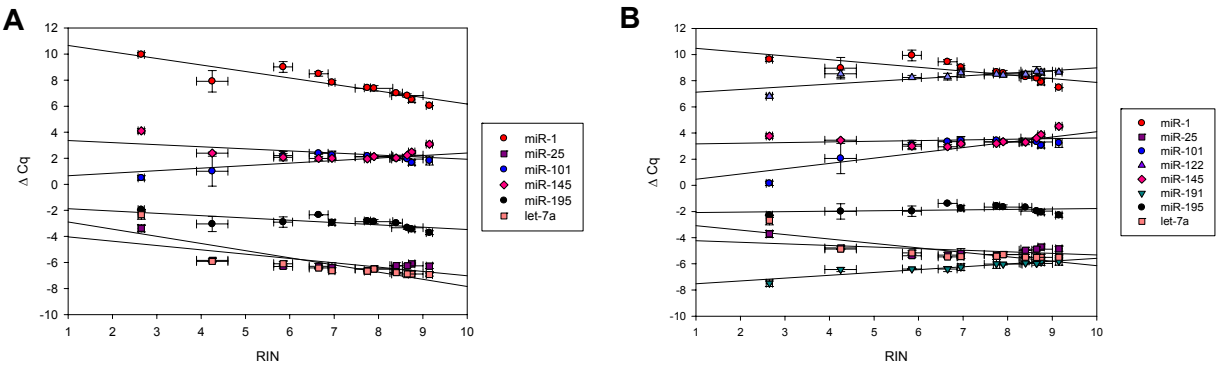

Figure 20: miRNA expression data for WBC normalized with an RG index (A) and the mean expression (B) value according to the ΔCt model

The positive impact of normalization on biased expression data was not as effective in miRNA as in mRNA. Despite normalization, for almost all results a statistically significant correlation between the RIN and the ΔCq value occurred.

To deal with factors adversly affecting the performance of RT-qPCR, it is important to apply a suitable normalization strategy in data analysis. The nomalizaton of expression data can partly reduce the impairing influence of RNA quality on the performance of RT-qPCR [101]. For mRNA, the use of an RG index calculated as the geometric mean of multiple RG is generally accepted [101]. By now, there is no universally valid guideline for normalization of miRNA expression data. In literature, different strategies are described. Other endogenous small RNAs (nuclear and nucleolar RNAs) are used as internal control and also universal “reference miRNAs” (miR-17-5p, miR-103, miR-191) have been described [103]. miR-191 is also defined as a proper normalizer by GenEx in the current study, but just for one tissue. This finding suggests that normalizers for miRNAs are tissue and species specific just like normalizers for mRNA studies. They should not be determined generally, but tested for each experiment separately. Recently published data proved that normalization using the mean expression value or stable endogenous miRNAs used similarly to the RG index showed the best reduction of technical variances in RT-qPCR data [102]. The mean expression value is mostly used for high-throughput experiments like microarrays or qPCR array setups, whereas an RG index serves as normalizer in experiments focusing on limited number of genes like RT-qPCR. Both methods have been tested for normalization of the expression data. Looking at the genes investigated in the actual experiment, the normalization with the mean expression value gave comparable results to the normalization using RG. For this case, we could show that the normalization using the mean expression value may also be

practicable for experiments with a limited number of genes and its application seems not just to be valid for profiling of large numbers of genes.

Obviously, the normalization of miRNA is much more sensitive to RNA quality than the normalization of mRNA. In spite of normalization, for almost all mesured miRNAs and all tissues a significant correlation of the RIN and the ΔCq remained. This result was comparable for normalization with an RG index or either the mean expression value. It is evident that the linearity is just interupted by outliers in the range of very low RNA quality (degradation step 10/11). Thus, the threshold of a RIN = 5 for gaining reliable PCR results, which was stated by Fleige et al. (2006) [34] could be confirmed for mRNA and also stated for miRNA by the actual study.

3.2.2 Changes in the miRNA expression profile under the influence of TBA plus E2 in bovine liver

Few is known about the expression of miRNAs in bovine species making it impossible to gain enough information from current scientific literature for adequate target gene search. Thus, a screening method has to be applied to identify changes in the miRNA expression. Human PCR arrays have been used as a screening method to find candidate miRNAs, as no species-specific arrays for cattle are commercially available, but miRNAs are known to be highly homologous between different species. These human Ready-to-use PCR Panels contain 742 confirmed human miRNAs.

On the array, 14 miRNAs were shown to be significantly up-regulated (table 12) and 22 miRNAs were shown to be significantly down-regulated (table 13) ($p<0.05$). In 14 miRNAs a trend for regulation could be observed ($p<0.1$), but these did not reach statistical significance.

Table 12: miRNA shown to be up-regulated in bovine liver under the influence of TBA+E2 on the PCR array (* = $p<0.05$; **= $p<0.01$; value 1.00 represents no expression change)

miR-	x-fold regulation ($2^{-\Delta\Delta Cq}$)	*p*-value	significance level
412	3.18	0.006	**
103-2*	2.16	0.010	**
15a	1.23	0.010	**
192*	1.72	0.029	*
378*	22.04	0.030	*
532-5p	1.38	0.027	*
103	1.20	0.032	*
885-5p	1.50	0.030	*
let-7f2	1.23	0.032	*
152	1.62	0.041	*
493	1.97	0.036	*
744*	1.71	0.046	*
215	1.61	0.049	*
572	39.49	0.054	*
138	3.42	0.060	
192	1.49	0.061	
1247	1.79	0.066	
194	1.50	0.087	
505	1.52	0.094	
146-5p	1.27	0.090	
125a-3p	4.43	0.089	

Table 13: miRNA shown to be down-regulated in bovine liver under the influence of TBA+E2 on the PCR array (* = p<0.05; **= p<0.01; ***= p<0.001; value 1.00 represents no expression change)

miR-	x-fold regulation ($2^{-\Delta\Delta Cq}$)	*p*-value	significance level
628-3p	0.15	0.001	***
130a	0.40	0.005	**
34a	0.31	0.006	**
29c	0.67	0.008	**
7-1*	0.57	0.016	*
29a	0.78	0.017	*
551b	0.32	0.017	*
765	0.29	0.018	*
132	0.56	0.022	*
29b	0.61	0.022	*
137	0.62	0.022	*
27b	0.53	0.022	*
98	0.74	0.026	*
181c	0.35	0.026	*
505*	0.05	0.027	*
708	0.41	0.033	*
455-5p	0.52	0.035	*
135a	0.37	0.037	*
486-5p	0.69	0.040	*
135a	0.36	0.040	*
433	0.40	0.046	*
518f	0.58	0.047	*
324-5p	0.82	0.057	
193-5p	0.73	0.065	
29c*	0.86	0.070	
126*	0.58	0.074	
93*	0.64	0.087	
494	0.57	0.089	
92a-1*	0.40	0.091	

In the miRNA screening, 3 randomly picked samples from the control and the treatment group, respectively, were investigated (n=6). The expression of prominent miRNAs, which might be candidates for physiological regulations, were validated in all experimental samples (n=18) using single assay PCR (table 14).

Table 14: Validation of prominent miRNA candidates using single assay PCR
(* = p<0.05; n.s. represents non-significant data; value 1.00 represents no expression change)

miR-	x-fold regulation ($2^{-\Delta\Delta Cq}$)		p-value	significance
	↑	↓		
15a		0.49	0.023	*
20a		0.49	0.024	*
27b		0.97	0.58	n.s.
29c	1.30		0.066	n.s.
34a		0.67	0.017	*
103	1.30		0.040	*
106a	1.40		0.057	n.s.
138	1.74		0.2	n.s.
181c		0.75	0.038	*
320d		0.72	0.065	n.s.
433	1.15		0.89	n.s.

Results from single assay PCR and PCR arrays have been correlated using linear regression (figure 21+ 22).

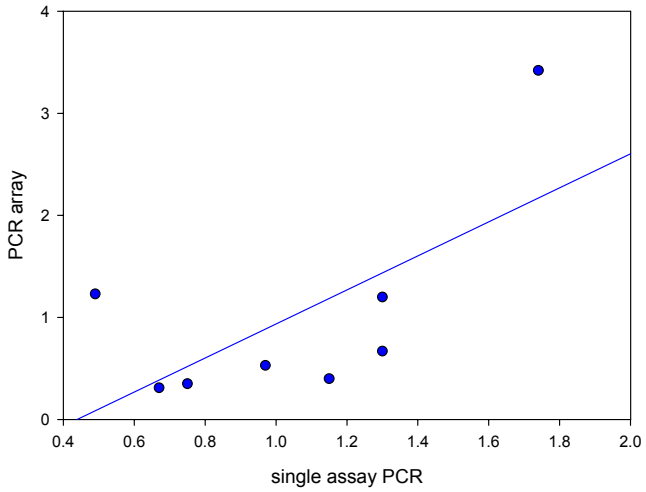

Figure 21: Correlated mean x-fold regulation ($2^{-\Delta\Delta Cq}$) from the single assay PCR (n=18) on the x-axis and the PCR array (n=6) on the y-axis with a slope of 1.668 and r^2=0.430 (p=0.077)

No satisfactory regression could be found comparing the mean x-fold expression from the PCR array (n=6) to the single assay PCR (n=18). Not only the statistical significance disappeared (e.g. miR-27b, miR-29c), which would be typical after taking a bigger cohort into

account due to introducing a higher inter-individual variance, but also the direction of the regulation changed in some cases from up- to down-regulation (e.g. miR-15a) or vice versa (e.g. miR-29c).

However, when correlating the single values for the x-fold expression of the PCR array results with the single assay qPCR results from these 6 samples, which were investigated in both cases, a highly significant linear regression could be found showing good reproducibility of gene expression data gained from single assay or array qPCR (figure 22).

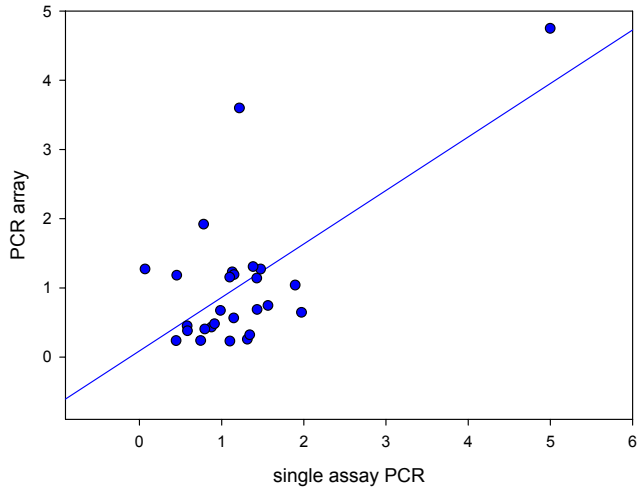

Figure 22: Correlated x-fold regulation ($2^{-\Delta\Delta Cq}$) of the control group compared to the treatment group in the 11 candidate miRNAs measured both on the PCR array and validated in single assay PCR with the single assay PCR data on the x-axis and the PCR array data on the y-axis with a slope of 0.773 and r^2=0.662 ($p<0.001$)

These observations show clearly that PCR arrays are a good approach for treatment screening. Herein, the total experimental cohort should be investigated on the array to gain valid results for physiological discussions. When measuring only random samples, the expression of prominent miRNA candidates should be validated by single assay PCR using the whole experimental cohort.

miRNAs exhibit regulatory effects on a variety of physiological processes in liver like proliferation and nutrient metabolism. Their dysregulation might be a key factor in different liver diseases [104]. Several studies showed aberrant expression of different miRNAs like miR-34a, miR-15a and miR-29c, in hepatocellular carcinoma (HCC) in comparison to normal cells. miR-34a has been demonstrated to be a direct transcriptional target of the pro-apoptotic tumor suppressor p53, as p53 binding sites have been detected in the miR-34a promotor region [105]. miR-34a down-regulation, which has been observed in further types of

cancer like neuroblastoma and pancreatic cancer cells, could partly be explained by the known inactivation of p53 in tumor cells. In the intact cell, p53 acts as a transcription factor and promotes the transcription of miR-34a. miR-34a represses the translation of anti-apoptotic factors like bcl-2 and therefore stimulates apoptosis [106]. The down-regulation of miR-34a (p=0.017) observed in the actual study, could indicate an inhibition of apoptosis and could account for the proliferative response following anabolic treatment.

Another factor associated with proliferation and tumorigenesis is miR-15a, which is a known tumor suppressor. Together with miR-16, miR-15a is implicated in the regulation of the cell cycle. Overexpression of these two miRNAs induces cell cycle arrest by inhibition of G1 cyclins and apoptosis by repression of the anti-apoptotic bcl-2 gene. Their dysregulation has been correlated with different pathophysiological events in liver, which are associated with uncontrolled proliferation, like HCC or polycycstic liver disease [104]. In the actual work, a down-regulation of miR-15a was detected (p=0.023). Also, aberrant expression of the miR-181 family has been implicated in tumor genesis [107]. miR-181c has been investigated in this thesis and has been shown to be significantly down-regulated (p=0.038). There are no data for miR-181c considering hepatocarcinogenesis, but its down-regulation has been detected in different kinds of gastric cancer. Herein, miR-181c has been shown to target different oncogenic factors and the missing repression would therefore lead to gastric carcinogenesis [108]. miR-181c might play a similar role in liver possibly leading to uncontrolled proliferation implicating the risk of tumorigenesis. The results for miR-15a and miR-181c fit in the observations from the other investigated tumorigenic and proliferative factors. Additionally, miR-103 (p=0.040) and miR-106a (p=0.057), which have both been found to be up-regulated, have been defined as oncogenic miRNAs inducing proliferation [104; 109]. It could be assumed that these regulations lead to an inhibition of apoptosis and a continued cell cycle resulting in proliferation and cell growth. miR-320, which showed a trend for down-regulation (p=0.065), is implicated in the regulation of IGF-1 mRNA expression by decreasing mRNA stability and inducing translational repression [110]. An up-regulation of IGF-1 could also be found on the mRNA level in this study [113]. These results indicate that the amount of IGF-1 mRNA is increased and additionally the half-life of this mRNA is expanded due to the down-regulation of miR-320. Therefore, the strong proliferative IGF-1 response due to the application of anabolic steroids seems to be mediated both on the transcriptional and the posttranscriptional level. Taken these results together, the observed changes in miRNA expresison indicate a trend versus proliferation in liver with a risk for tumorigenesis due to uncontrolled cell growth after the application of anabolic steroid

hormones [54; 57; 111]. The same conclusions have already been drawn from regulations on the mRNA level [113].

The trend for up-regulation of miR-29c ($p=0.066$) does not fit into the picture, which is drawn by the other regulated miRNAs, as this miRNA exhibits tumor suppressive action. Its down-regulation has been detected in hepatocarcinogenesis [112]. Xiong et al. revealed the important role of miR-29c in the induction of apoptosis by the repression of the anti-apoptotic bcl-2 gene. The down-regulation of miR-29c in tumor cells is part of the strategy to circumvent apoptosis and to survive in the tumor environment often showing low nutrient status and hypoxia. HCC cells have also been shown *in vitro* to be sensitized to apoptosis after reintroduction of miR-29c. Herein, miR-29c causes an up-regulation of p53 and induces apoptosis in a p53-dependent manner [112]. So possibly, the trend for up-regulation observed in the actual investigation might be the beginning of an adaption mechanism of the organism to prevent the negative side effects of anabolic treatment leading to uncontrolled proliferation. The increased amounts of miR-29c could restrict the p53 dependent down-regulation of miR-34a and the following inhibition of apoptosis and might sensitize aberrant hepatocyctes to apoptosis. It has to be kept in mind, that the analysis of the transcriptome is always just a snap-shot of the actual situation and it might be possible that this adaption mechanism may become manifest later on. However, taking the regulations present at the certain time point and results from mRNA expression analysis into account, a trend for cell growth by the induction of proliferative factors and an inhibition of apoptotic factors is assumed. Additionally, the up-regulation of miR-29c could display another function of the miR-29 family. In diabetes research, the miR-29 family has been implicated in the repression of insulin-stimulated glucose uptake in storage organs. mRNA expression analysis indicated a lower insulin responsiveness of the liver due to the down-regulation of both IRα and IRβ. These observations on mRNA and miRNA level indicate a lower glucose uptake into the liver. Thus, glucose would remain in the blood flow and could be used as energy substrate in other target organs e.g. muscle cells for proliferative processes due to anabolic treatment.

PCA of gene expression data has been produced as shown in figure 23 (A+B). Animals from the control group are represented by grey squares, animals from the treatment group are represented by black circles. Gene expression data from regulated miRNAs from single assay PCR have been plotted (figure 23A).

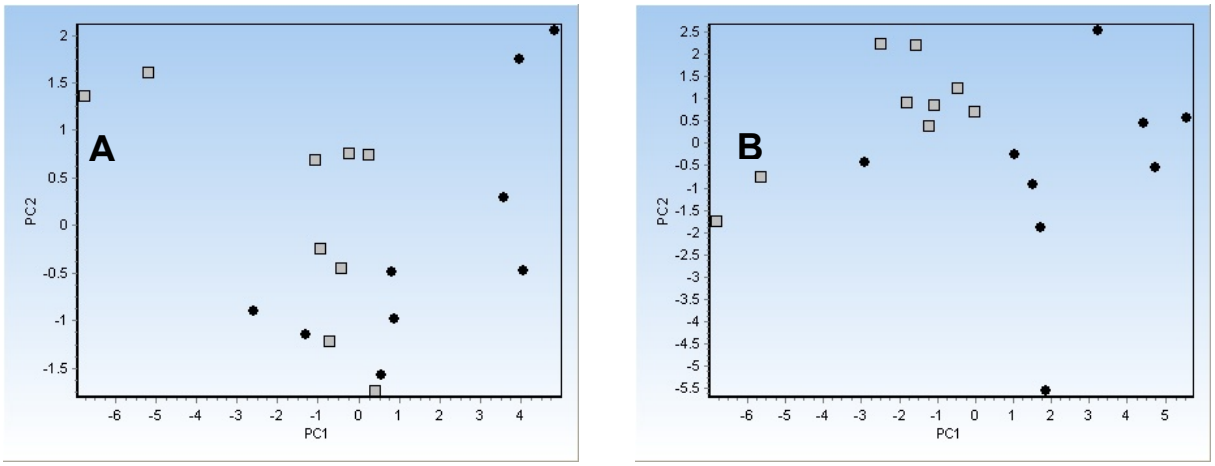

Figure 23: PCA of 8 regulated miRNAs from single assay qPCR (A) and of 8 regulated target genes from miRNA single assay PCR plus 15 regulated target genes from mRNA expression analysis (B)

Herein, a slight separation could be observed between the control and the treatment group for regulated miRNA expression results from single assay PCR. This indicates that the measured miRNA pattern is not yet sufficient to differentiate between treated and non-treated animals. It might be possible to reach a better separation by integration of more target genes. Additionally, regulated miRNAs have been combined with regulated target genes from mRNA expression analysis from the same transcriptome (figure 23B) [113]. In this PCA, a clear differentiation between the animals from the control and treatment group could be observed with just one animal from the control group being expresses in a similar range like the animals of the treatment group.

These results indicate that the combination of miRNA and mRNA expression results in data evaluation using bio-statistical methods like PCA might be a promising approach to carry on biomarker research and to find a general gene expression pattern for anabolic treatment screening.

4 Conclusions and perspectives

Changes in gene expression have been investigated in liver and reproductive tract of bovine and a variety of transcriptional regulations could be detected for genes involved in several biochemical pathways on the mRNA as well as on the miRNA level after the application of anabolic steroids. A summary of all genes regulated in the two animal studies included in this thesis is displayed in figure 24.

These results helped significantly to understand the physiological changes in the transcriptome leading to the known phenotypes of the respective target organs following anabolic treatment. In general, the regulations on gene expression level indicate a trend for local and peripheral growth processes. Increased local cell turnover was characterized by the stimulation of proliferation and inhibition of apoptosis. Peripheral growth processes were induced by the supply of growth factors (e.g. IGF-1) and energy substrates (e.g. glucose).

For the first time, the miRNA expression profile has been examined under the influence of anabolic steroids in bovine species. This analysis was challenging as few was known about miRNA expression in cattle and no validated assays were available for amplification of bovine miRNAs. Prior to physiological investigations, an extraction method and a valid qPCR assay including a reliable normalization method were established. It was proven that human miRNA assays for qPCR could be used in bovine samples for sequences showing 100% homology. The influence of RNA degradation on gene expression measurement, which had by now only been investigated for mRNA, was estimated for miRNA and could be considered to gain valid results.

The physiological discussions drawn for mRNA could be supported by changes in the miRNA expression in liver of Nguni heifers showing distincly that also changes in the miRNA expression could be a good indicator for anabolic treatment.

Furthermore, the application of RT-qPCR in combination with appropriate bio-statistical tools for pattern recognition has been proven to be a suitable approach to find gene expression profiles, which might be used for anabolic treatment screening.

In this thesis, PCA has been the method of choice for pattern recognition for all gene expression results and demonstrated clearly the possibility to establish a gene expression profile in all investigated tissues. A distinct separation between control and treated animals could be shown in liver, uterine endometrium and ovary of Nguni heifers. Herein, the best separation could be observed in the combination of uterine endometrium and ovary. So, the whole reproductive tract seems to be the most auspicious tissue for biomarker research on mRNA level.

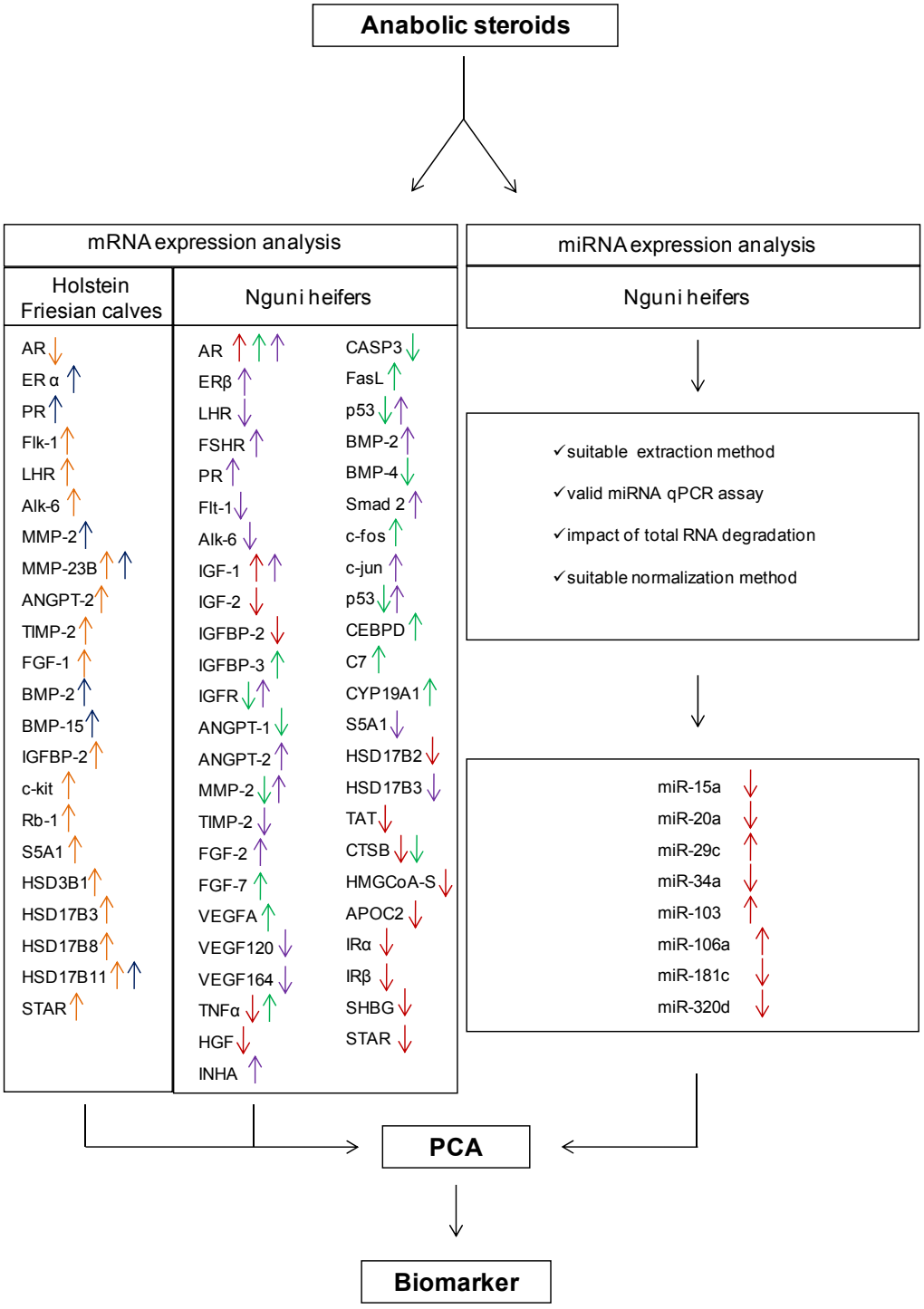

Figure 24: Summarized results of the thesis, arrows represent the direction of the regulation; ↑ stands for an up-regulation, ↓ stands for a down-regulation; different colors represent different tissues or treatment groups; in Holstein Friesian calves orange arrows display animals from the 1x treated group, blue arrows display animals from 3x treated group; in Nguni heifers, red arrows represent results from liver, green arrows represent results from uterine endometrium and purple arrows represent results from ovary

A limitation in using the reproductive tract is that endometrium and ovary are only available from female animals. So, further investigations need to include prostate and testis tissue and should confirm the suitability of reproductive organs also for male animals. Besides other tissues, bigger experimental cohorts should be integrated in animal studies. Especially the number of control animals should be increased to introduce a higher biological variability to fortify the suitability of gene expression patterns for the use as biomarker.

Unfortunately, no separation could be observed for ovary of Holstein Friesian calves. For this tissue, also in gene expression analysis divergent results have been obtained between heifers and calves under the influence of anabolic combinations of androgens and estrogens. Possible reasons for these differences have been deeply discussed and could be due to hormonal status and age class (pre- versus post-pubertal) or treatment regime (permanent implant versus pour on treatment). Thus, pre-pubertal animals, the choosen gene expression pattern or the treatment regime (pour on treatment) seem to be less suitable for surveillance of anabolic treatment via gene expression measurement.

Considering miRNA, the best separation could be reached when combining results from mRNA and miRNA from the same transcriptome indicating that the combination of gene expression results from mRNA and miRNA seems to be a promising approach to find a gene expression pattern, which might be used for anabolic treatment screening.

miRNA expression patterns have been profoundly investigated in tumorigenesis and are up-coming biomarkers in early diagnosis of different kind of cancer. Due to their tissue- and diesease-specificity, miRNA profiles have proven their superiority over mRNA expression patterns. The investigation of transcriptional changes on the miRNA level and also the combination of miRNA with mRNA expression results might be a new and precise approach for surveillance of anabolic treatment. Potentially, a treatment specificity of the miRNA expression pattern could be proven for anabolic treatment as disease specificity has been shown in cancer diagnosis.

For ongoing research on the influence of anabolic steroid hormones on miRNA expression, valid screening methods should be applied, as few is known about the physiological functionality of miRNAs in cattle. In the current thesis, PCR arrays have been used for screening and have been shown to be a valid and cost-effective method compared to other screening strategies like Next Generation Sequencing (NGS) or microarrays. However, especially NGS would give a hypothesis-free view over the whole transcriptome including mRNA and regulatory small RNA providing holistic information on gene expression. Further research should also focus on the discovery of new bovine miRNA and the revealing of physiological functions of miRNAs in the bovine organism.

The results of this thesis show clearly the potential of mRNA plus miRNA for biomarker development to discover misuse of anabolics. However, further research is needed to confirm the results from the actual thesis and to estimate the influence of breed, gender, nutrition, hormonal status or the treatment regime. Potentially, different tests have to be evaluated for different age classes or anabolic drug combinations.

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"mRNA and microRNA quality control for RT-qPCR analysis"
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J Chromatogr A. 2009 Nov 13; 1216(46):8192-9. Epub 2009 Feb 5

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"Design and optimization of reverse-transcription quantitative PCR experiment"
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"Detectability of testosterone esters and estradiol benzoate in bovine hair and plasma following pour on treatment"

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Book Chapters:

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Application notes/short communications:

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Appendix

Appendix I:

Becker C, Hammerle-Fickinger A, Riedmaier I, Pfaffl MW

"mRNA and microRNA quality control for RT-qPCR analysis"

Methods, Special Issue: The ongoing evolution in qPCR, 2010 Apr; 50 (4):237-43

Appendix II:

Becker C, Riedmaier I, Reiter M, Tichopad A, Pfaffl MW, Meyer HHD

"Effect of trenbolone acetate plus estradiol on transcriptional regulation of metabolism pathways in bovine liver"

HMBCI, 2010, 2 (2): 257-265

Appendix III:

Becker C, Riedmaier I, Reiter M, Tichopad A, Groot MJ, Stolker AAM, Pfaffl MW, Nielen MFW, Meyer HHD

"Influence of an anabolic combination of an androgen and an estrogen on biochemical pathways in bovine uterine endometrium and ovary"

JSBMB, submitted

Appendix IV:

Stolker AA, Groot MG, Lasaroms JJ, Nijolder AW, Blokland MH, Riedmaier I, Becker C. Meyer HH, Nielen MW

"Detectability of testosterone esters and estradiol benzoate in bovine hair and plasma following pour on treatment"

Anal Bioanal Chem. 2009 Oct; 395 (4): 1075-87

Appendix V:

Riedmaier I, Becker C, Pfaffl MW, Meyer HHD

"The use of omic technologies for biomarker development to trace functions of anabolic agents"

J Chromatogr A. 2009 Nov 13; 1216 (46):8192-9. Epub 2009 Feb 5

Appendix VI:

Becker C, Riedmaier I, Reiter M, Pfaffl MW, Meyer HHD

"Changes in the miRNA profile under the influence of anabolic steroids"

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Appendix I

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mRNA and microRNA quality control for RT-qPCR analysis

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ABSTRACT

The importance of high quality sample material, i.e. non-degraded or fragmented RNA, for classical gene expression profiling is well documented. Hence, the analysis of RNA quality is a valuable tool in the preparation of methods like RT-qPCR and microarray analysis. For verification of RNA integrity, today the use of automated capillary electrophoresis is state of the art. Following the recently published MIQE guidelines, these pre-PCR evaluations have to be clearly documented in scientific publication to increase experimental transparency.

RNA quality control may also be integrated in the routine analysis of new applications like the investigation of microRNA (miRNA) expression, as there is little known yet about factors compromising the miRNA analysis. Agilent Technologies is offering a new lab-on-chip application for the 2100 Bioanalyzer making it possible to quantify miRNA in absolute amounts [pg] and as a percentage of small RNA [%]. Recent results showed that this analysis method is strongly influenced by total RNA integrity. Ongoing RNA degradation is accompanied by the formation of small RNA fragments leading to an overestimation of miRNA amount on the chip. Total RNA integrity is known to affect the performance of RT-qPCR as well as the quantitative results in mRNA expression profiling. The actual study identified a comparable effect for miRNA gene expression profiling. Using a suitable normalization method could partly reduce the impairing effect of total RNA integrity.

1. Introduction

The expression level of RNAs serves as a good indicator of the physiological status of a cell or tissue. Various studies showed a distinct influence of total RNA integrity on the performance of gene expression profiling using RT-qPCR or microarrays [1–3]. RNAs are very sensitive molecules and the ubiquitous occurrence of nucleases poses a constant risk of RNA degradation. For this reason cautious handling in every single pre-PCR step of the gene expression analysis (e.g. sampling, storage and extraction) is important as only experiments conducted with high quality starting material provide reliable results. The recently published guidelines for "minimum information for publication of quantitative real-time PCR experiments" (MIQE guidelines) demand a higher transparency of the pre-PCR steps like the documentation of sample quality [4]. These guidelines are supposed to give recommendations for authors, which details are necessary to be declared in a publication. This should guarantee to get a standardized paperwork for gene expression experiments to help the reader to evaluate and reproduce published results, to promote consistency between laboratories, and to increase experimental transparency.

1.1. Total RNA quality control

RNA quality control arose the interest in gene expression analysis as it was shown to strongly influence the performance and quantitative data of RT-qPCR, which is the method of choice to study gene regulation. The term RNA quality is defined as the composition of RNA purity and RNA integrity.

1.1.1. RNA purity

RNA purity can be measured photometrically using the NanoDrop (peqLab Biotechnologie GmbH, Erlangen, Germany), the NanoVue (GE Healthcare, Munich, Germany) or other sensitive spectrophotometers e.g. the NanoPhotometer (Implen, Munich, Germany), which is an optimal solution for application of very small volumes. The optical density (OD) is measured at different wave lengths: 230 nm (absorption of contaminants & background absorption), 260 nm (absorption maxima of nucleic acids), 280 nm (absorption maxima of proteins), and 320 nm (absorption of contaminants & background absorption). The $OD_{260/280}$ ratio is used as indicator for RNA purity. A ratio higher than 1.8 is assumed as suitable for gene expression measurements [5,6]. The $OD_{260/230}$ and the $OD_{260/320}$ should be maximized as these represent the degree of background absorption and contaminants.

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Classical quality control of nucleic acids uses high resolution 4% agarose gel electrophoresis to separate the different fractions (5S, 18S, 28S) of ribosomal RNA (rRNA) subunits. For RNA of good quality a 28S/18S ratio of 2.0 is assumed. The subjective interpretation of these agarose gel images strongly depends on the experience and examination of the individual researcher and can hardly be compared between different users and laboratories.

1.1.2. Total RNA integrity control

Today, lab-on-chip technology for automated capillary electrophoresis is state of the art and is recommended for standardized RNA integrity control. Different lab-on-chip instruments are commercially available like the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and the Experion (Bio-Rad Laboratories, Munich, Germany). Both devices are sensitive, highly reproducible and suitable for a reliable quality control of RNAs [19]. For visualization and better interpretation, an electropherogram and a virtual gel image are generated. The 28S/18S ratio is calculated by assessing the peaks recorded in the electropherogram and the bands occurring on the gel-like image. Additionally, to simplify the assessment of RNA integrity the instrument software calculates a numerical value: RNA integrity number (RIN) on the 2100 Bioanalyzer and RNA quality index (RQI) on the Experion. A RQI/RIN of 1 represents almost fragmented and degraded RNA and a RQI/RIN of 10 represents intact and non-fragmented RNA [7].

1.2. Quality control in miRNA analysis

MicroRNAs (miRNAs) are small RNAs with a length of approximately 22 nucleotides, those are thought to be involved in the regulation of many physiological processes like growth and development. These molecules were already described in 1993 [8], the name "miRNAs" was primary alluded in 2001, and the analytical interest in valid miRNAs quantification arose over the past years. Concerning functional studies, especially the investigation of miRNA expression profiles is of great interest, because miRNAs are implicated in the genesis of different cancer types and therefore could be used as clinical markers in diagnosis [9–11]. As miRNAs belong to the group of nucleic acids, they are examined with the same technologies as long RNAs like mRNAs. Problems start with the quantification and quality control of miRNAs, as classical photometrical methods for measuring the concentration of nucleic acids do not allow discriminating between different fractions of RNAs. For quantitative expression profiling of mRNAs, RT-qPCR has become the gold standard. Concerning mRNA, factors influencing RT-qPCR like inhibitors or RNA quality are well investigated and the immane influence of RNA integrity on the performance of RT-qPCR and quantitative results is stated [1,2,12]. The evaluation of RNA integrity should also be integrated as a routine step in pre-PCR for expression profiling of miRNAs, as little is known about the accessibility of miRNA to degradation and the influence of total RNA integrity as a factor possibly compromising the expression profiling of miRNAs [13]. Agilent Technologies offers a new small RNA tool on the 2100 Bioanalyzer making it possible to analyze small RNA (<200 nt) with the lab-on-chip technology. Within this small RNA fraction, fragments with a size of 15–40 nt are defined as miRNA (Fig. 1A). The concentration of miRNA is calculated as absolute amount [pg] and as a percentage of small RNA [%]. By now, this chip offers one of the few possibilities to quantify miRNA.

1.3. Aim of the current study

A study was conducted to investigate the influence of total RNA quality on mRNA and miRNA quantification with the small RNA Assay on the Bioanalyzer and the miRNA expression measured using RT-qPCR. Also, an adequate normalization method for miRNA expression data should be validated, as normalization is an essential step in RT-qPCR analysis to avoid technical variations and to prove that the evaluated miRNA expression differences are of biological kind.

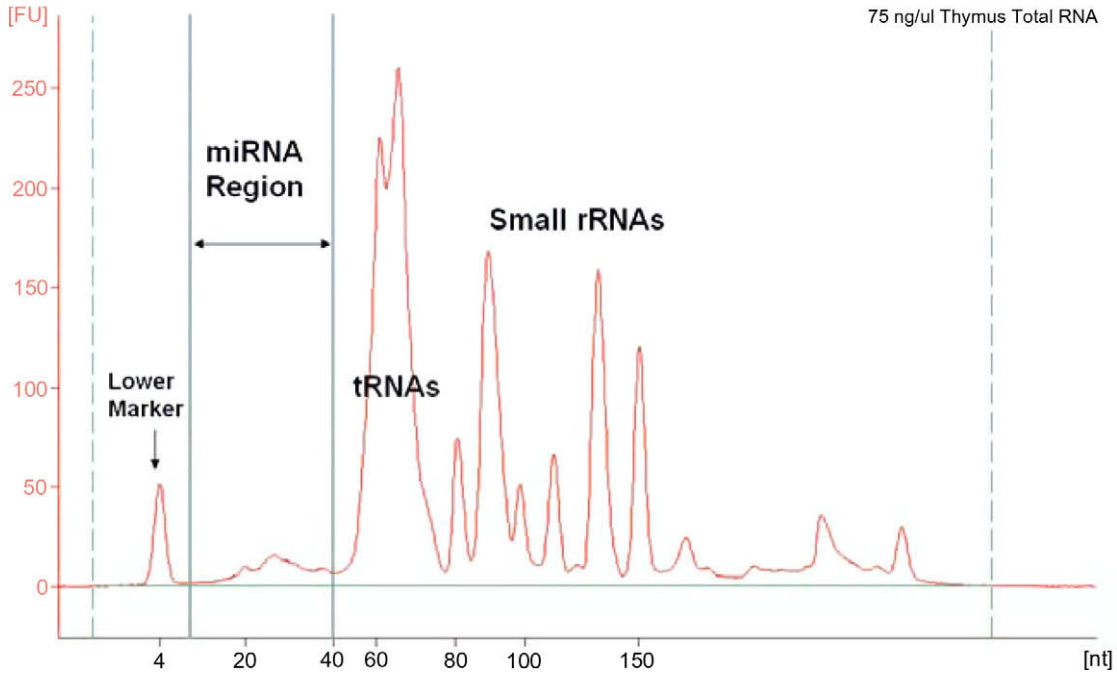

Fig. 1A. Image of a typical electropherogram for small RNA analysis performed with the Small RNA Assay on the 2100 Bioanalyzer (Agilent Technologies) (http://www.chem.agilent.com/Library/technicaloverviews/Public/5989-7002EN.pdf).

2. Description of methods

2.1. RNA extraction

Total RNA has been extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. Extractions were done from different bovine tissues [liver, muscle, white blood cells (WBC)] in six replicates per tissue ($n = 6$).

2.2. RNA degradation

For artificial RNA degradation, the six replicates of each tissue were pooled and the pool divided into two equal portions. One portion was degraded by exposure to UV light for 90 min to create a fragmented and degraded RNA fraction. The second portion remained untreated and served as intact and non-degraded RNA fraction. To create a linear gradient between intact RNA and degraded RNA from the identical transcriptome, the two fractions were mixed in changing ratios. A serial dilution with 11 degradation steps was created, whereby step 1 being intact RNA (consisting of 100% intact RNA, 0% fragmented RNA) going down in 10% steps with the intact RNA to step 11 being the most degraded RNA (consisting of 0% intact RNA; 100% fragmented RNA). This was done for all three tissues separately.

2.3. RNA quantification and RNA integrity control

Total RNA concentration has been quantified with the NanoDrop ND-1000 (peqLab Biotechnologie GmbH) by measuring the extinction at 260 nm. Additionally, the $OD_{260/230}$ and the $OD_{260/280}$ ratio showing RNA purity were examined. Quality control has been done with the 2100 Bioanalyzer using "Eukaryote total RNA Nano Assay" (Agilent Technologies). The RNA integrity number (RIN) served as RNA integrity parameter. Quantification and quality assessment of small RNA including the miRNA fraction were undertaken with the "Small RNA Assay" (Agilent Technologies). All chips were done as duplicates.

2.4. Primer design

Primer pairs for mRNA expression (Table 1) analysis were either newly designed using published bovine nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or previously established primer sequences were used. Newly designed primers were ordered and synthesized at MWG (Ebersberg, Germany).

"miScript Primer Assays" for specific miRNA targets were synthesized and ordered at Qiagen. As no specific bovine primer assays are available, human assays were used after checking sequence homology using http://www.mirbase.org/. The investigated miRNAs showed 100% homology between human and bovine sequences, except miR-195, which showed a single nucleotide aberration at the 3′-end.

2.5. Reverse transcription

RNA samples were converted to cDNA using MMLV H^{minus} reverse transcriptase (Promega, Regensburg, Germany). Therefore, 500 ng total RNA were diluted to a final volume of 13 μL. The master mix for the reverse transcription was prepared as follows: 4 μL 5× reaction buffer (Promega), 1 μL random primers (Invitrogen, Karlsruhe, Germany), 1 μL dNTP (Fermentas, St. Leon-Rot, Germany), 1 μL MMLV H^{minus} reverse transcriptase (Promega). After adding 7 μL of the mastermix to the diluted sample the plate was inserted in the Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany) and the here stated temperature protocol was started: 21 °C, 10 min; 48 °C, 50 min; 90 °C, 2 min; 4 °C hold. After reverse transcription, all samples were diluted to a final volume of 60 μL.

miRNA samples were poly-adenlyated, elongated and reverse transcribed in a separated step using the "miScript" system (Qiagen). The following mastermix: 2 μL 5× miScript buffer, 1 μL miScript reverse transcriptase was mixed with 500 ng total RNA diluted to a volume of 7 μL to a final volume of 10 μL. The plate was inserted in the Eppendorf Gradient Cycler (Eppendorf) and the here stated temperature protocol was started: 37 °C, 60 min; 95 °C, 5 min; 4 °C hold. After reverse transcription, all samples were diluted 6-fold to a final volume of 60 μL.

2.6. Quantitative PCR

Quantitative PCR (qPCR) was performed in the Realplex ep gradient S Mastercycler (Eppendorf). For automation, pipetting was done with the epMotion 5075 LH pipetting robot (Eppendorf). For mRNA, RealMasterMix SYBR ROX (5Prime, Hamburg, Germany) was used by a standard protocol recommended by the manufacturer. The mastermix was prepared as follows: 7.5 μL 2× RealMasterMix SYBR ROX, 0.75 μL forward primer (10 pmol/μL), 0.75 μL reverse primer (10 pmol/μL), 4.0 μL RNAse free water (5Prime). 13 μL of the mastermix were filled in a well and a 2 μL volume of 12.5 ng cDNA was added for a total volume of 15 μL. The qPCR protocol was started: denaturation step (94 °C, 2 min), cycling program (95 °C, 5 s; annealing temperature according to Table 1 and 10 s; 68 °C, 20 s) and melting curve analysis.

qPCR for miRNA was done using "miScript" system (Qiagen) according to the manufacturer's recommendation. The following

Table 1
Primer sequences used for mRNA expression analysis with gene name, sequence (3′ → 5′), annealing temperature (T_M), product length and accession number.

Gene	Sequence		T_M [°C]	Product length [bp]	Accession number
Ubiquitin	for	AGA TCC AGG ATA AGG GAA GGC AT	60	198	Z18245
	rev	GCT CCA CCT CCA GGG TGA T			
p53	for	ATT TAC GCG CGG AGT ATT TG GAC	60	174	NM_174201
	rev	CCAGTGTGATGATGGTGAGGA			
LDH	for	GTG GCT TGG AAG ATA AGT GG	60	155	NM174099
	rev	ACT AGA GTC ACC ATG CTC C			
Caspase 3	for	GAC AGT GGT GCT GAG GAT GA	60	164	NM_001077840
	rev	CTG TGA GCG TGC TTT TTC AG			
ACTB	for	AAC TCC ATC ATG AAG TGT GAC	60	202	AY141970
	rev	GAT CCA CAT CTG CTG GAA GG			
IL-1β	for	TTC TCT CCA GCC AAC CTT CAT T	60	198	M37211
	rev	ATC TGC AGC TGG ATG TTT CCA T			
Histon H3	for	ACTGCTACAAAAGCCGCTC	60	233	NM_001034034
	rev	ACTTGCCTCCTGCAAAGCAC			

Table 2
RIN values and miRNA quantification data from Bioanalyzer exemplary for WBC.

Good quality RNA [%]	Mean RIN	SD RIN	Mean miRNA/small RNA ratio [%]	SD ratio [%]	Mean miRNA concentration [pg/μL]	SD miRNA concentration [pg/μL]
100	9.15	0.07	3.80	0.45	592.54	17.76
90	8.75	0.07	4.54	0.66	765.47	69.21
80	8.65	0.35	5.69	0.54	1031.72	222.49
70	8.40	0.14	5.74	1.65	1141.24	129.56
60	7.90	0.42	7.59	0.37	1455.04	380.61
50	7.75	0.07	8.59	0.31	1643.46	563.81
40	6.95	0.07	10.49	0.44	1586.82	475.17
30	6.65	0.21	11.52	0.20	1897.65	773.89
20	5.85	0.21	11.69	0.44	1805.73	339.21
10	4.25	0.35	10.57	3.78	1623.96	808.92
0	2.65	0.07	13.11	0.74	2053.08	47.11

mastermix was prepared with all necessary components for PCR: 10 μL 2× QuantiTect SYBR Green PCR mastermix, 2 μL 10× universal primer, 10× miScript primer assay, 4 μL RNAse free water. Eighteen microliters of the prepared mastermix were filled in a well and 2 μL template from miRNA reverse transcription were added for a total volume of 20 μL and the following PCR protocol was started: denaturation step (95 °C, 15 min), cycling program (95 °C, 15 s; 55 °C, 30 s; 70 °C, 30 s) and melting curve analysis.

2.7. Data analysis

Quantification and expression data were statistically processed with SigmaStat 3.0 (SPSS, Chicago, IL). The determined *p*-values of the statistical significance were examined using linear regression and coefficient determination (r^2). Significance of linear regression was analyzed by Students's *t*-test by testing the slope to be different from zero. Level of significance was set for $p < 0.05$. All data were graphically plotted using SigmaPlot 11.0 (SSPS).

Suitable reference genes (RG) for normalization of gene expression data for mRNA and miRNA were evaluated by Cq stability and variability testing using the GenNorm and Normfinder algorithm in GenEx v. 4.3.6 (MultiD Analyses AB, Gothenburg, Sweden). The geometric mean of two RG was used as reference index.

Data were normalized according to the ΔCq model [14] with the following formula:

$$\Delta Cq = Cq_{(\text{target gene})} - Cq_{(\text{reference index})}$$

3. Results

3.1. RNA degradation

RNA degradation via UV light was successful in all tissues and a quality gradient could be created (exemplary the results for WBC are shown in Table 2 and Fig. 1B). As RNA quality consists of RNA integrity and RNA purity, also the $OD_{260/280}$ ratio and the $OD_{260/230}$ ratio have been checked photometrically to ensure good RNA purity. This examination showed constant RNA purity for all degradation steps with a mean $OD_{260/280}$ of 2.033 ± 0.027 and a mean $OD_{260/230}$ of 1.925 ± 0.14 ($n = 28$) indicating that the different degradation levels are solely caused by a shift in RNA integrity.

3.2. miRNA quantification

miRNA quantification using the 2100 Bioanalyzer "Small RNA Assay" showed a clear relation between the miRNA amount and

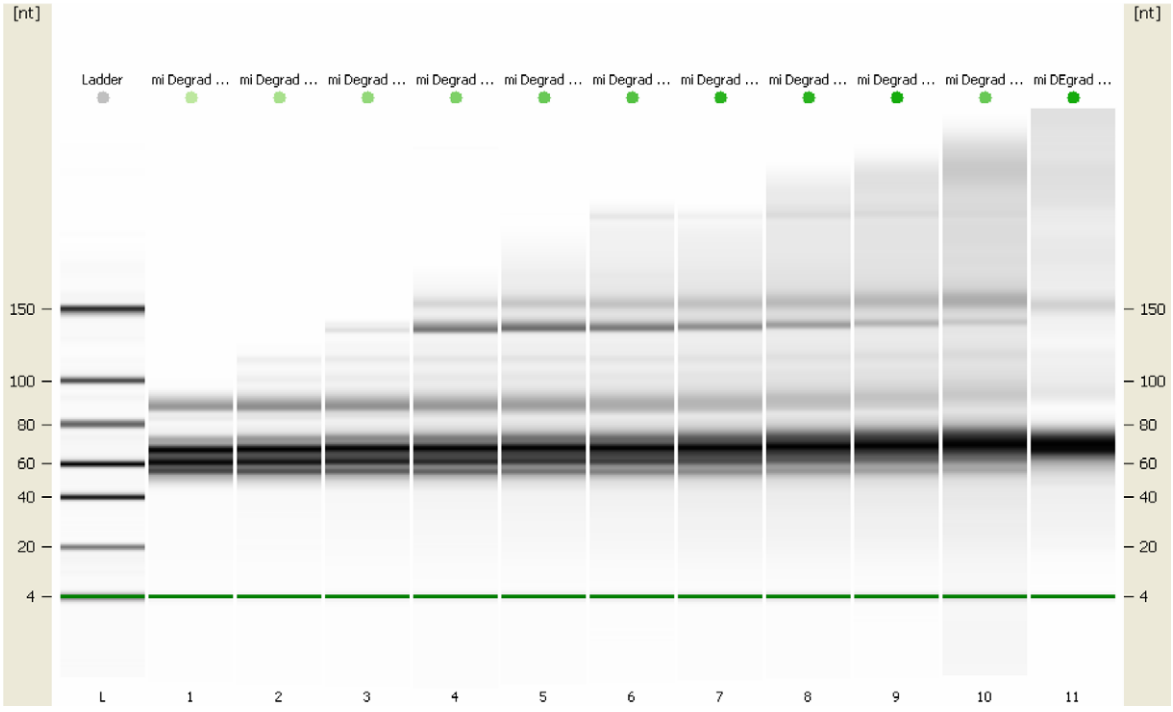

Fig. 1B. Electronic gel image on the small RNA assay from the results of UV-based degradation of WBC.

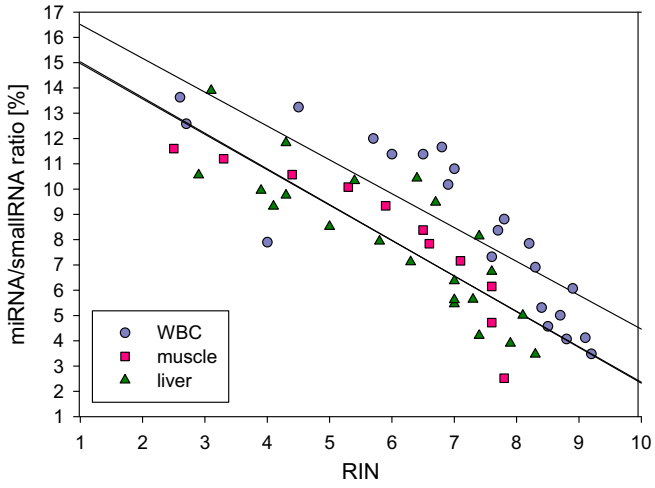

Fig. 2. Highly significant correlation between RIN and miRNA/small RNA ratio for liver, muscle and WBC with $p < 0.001$ for all subsets (regression lines for liver and muscle are overlapping).

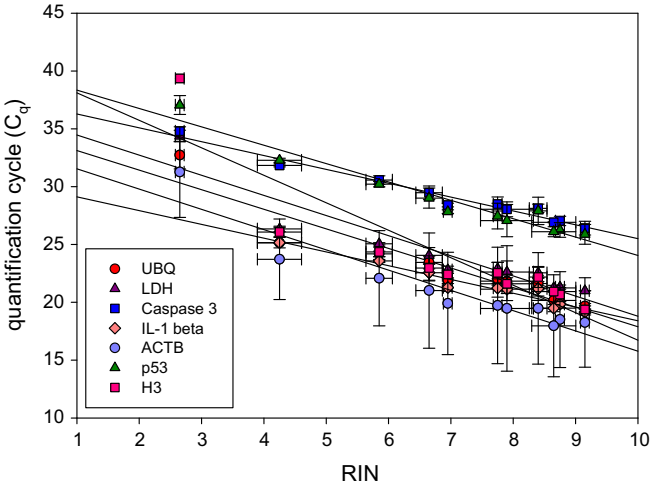

Fig. 3. mRNA expression data obtained from WBC showing a highly significant correlation between RIN and Cq value with $p < 0.001$ for all subsets.

the state of degradation for all tissues. With ongoing RNA degradation a significant rise ($p < 0.001$) in miRNA/small RNA ratio appeared for all tissues (Fig. 2). In liver and WBC, also a significant increase ($p < 0.001$) in the miRNA concentration occurred. An increase could also be shown for muscle tissue, but the rise was not statistically significant.

In WBC, the miRNA/small RNA ratio ascended from 3.80 ± 0.45% to 13.11 ± 0.74% showing a clear increase due to the formation of short RNA fragments during RNA degradation. Similar results were acquired for WBC and muscle. For muscle, an increase from 2.19 ± 0.47% to 11.08 ± 0.74% and for liver, an increase from 3.69 ± 0.30% to 12.23 ± 2.36% was shown. A comparable relationship was obtained for the correlation between RIN and miRNA concentration ($p < 0.001$). The miRNA concentration in liver rose from 999.92 ± 43.63 pg/μL to 2697.35 ± 616.21 pg/μL, in muscle from 814.49 ± 163.73 pg/μL to 2902.54 ± 306.89 pg/μL and in WBC from 592.54 ± 17.76 pg/μL to 2053.08 ± 47.11 pg/μL. An exemplary summary of the miRNA quantification results for WBC on the 2100 Bioanalyzer is stated in Table 2.

3.3. Results of gene expression analysis

Gene expression of mRNA and miRNA was measured using RT-qPCR. As expected, a distinct and highly significant, negative correlation between RIN and quantification cycle (Cq) could be shown for mRNA in all tissues and for all quantified genes (r^2_{mean}= 0.837; $p < 0.001$) with a mean slope of the regression line of 1.578. Exemplary, the gene expression results for WBC are demonstrated in Fig. 3. Analog results were obtained for muscle and liver tissue.

Comparable to mRNA expression, a highly significant correlation between the RIN and the Cq value ($r^2_{mean} = 0.835$; $p < 0.001$) could also be observed for miRNA expression with a mean slope of the regression line of 0.784. Expression results for WBC are shown in Fig. 4.

3.4. Normalization of expression data

mRNA expression data have been normalized using a reference index consisting of the geometric mean expression of two suitable RG, which were determined for every tissue by GenEx software. Optimal number of RG was selected using pairwise variation analysis integrated in geNorm algorithm implemented in GenEx [15]. For WBC, βActin (ACTB) and Lactatdehydrogenase (LDH); for muscle tissue, Ubiquitin (UBQ) and ACTB and for liver tissue, ACTB and Caspase 3 were used for calculation of an RG index. Normalized expression data were linearly regressed with the RIN. For almost all data no statistically significant correlation between the RIN and the ΔCq value could be shown. Significant correlations were consistent after normalization in muscle for Caspase 3 and IL-1β, in liver for UBQ and IL-1β and in WBC for Caspase 3 and Histon H3. For these genes, the correlation was no more significant, when elimination the results from degradation step 10 and 11 (10% and 0% good quality RNA), clearly showing that samples with a very low RIN are not suitable for qPCR analysis.

Two strategies were applied for normalization of miRNA expression data. Similar to the determination of RG for mRNA, suitable RG for miRNA were detected using GenEx software. For WBC, miR-122 and miR-191, for muscle and liver tissue, miR-122 and let-7a were used for calculation of an RG index [15].

As second strategy, normalization using the geometric mean expression value of all measured miRNAs was applied [18]. Examples for both normalization methods obtained in WBC are shown in Figs. 5 and 6.

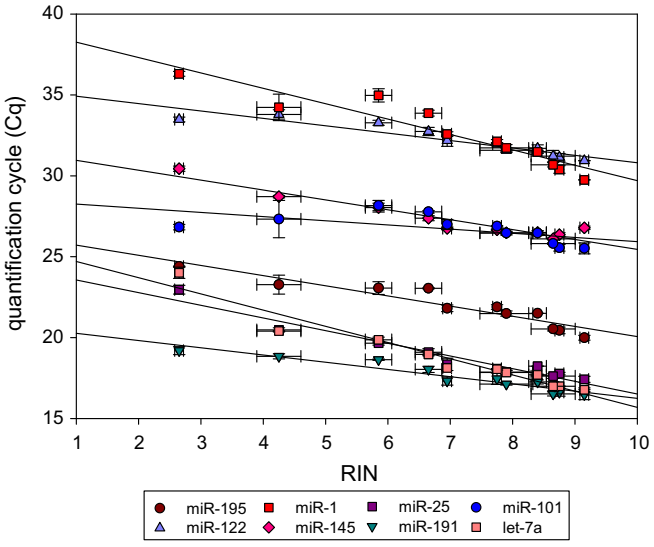

Fig. 4. miRNA expression data obtained from WBC showing a highly significant correlation between RIN and Cq value with $p < 0.001$ for all subsets.

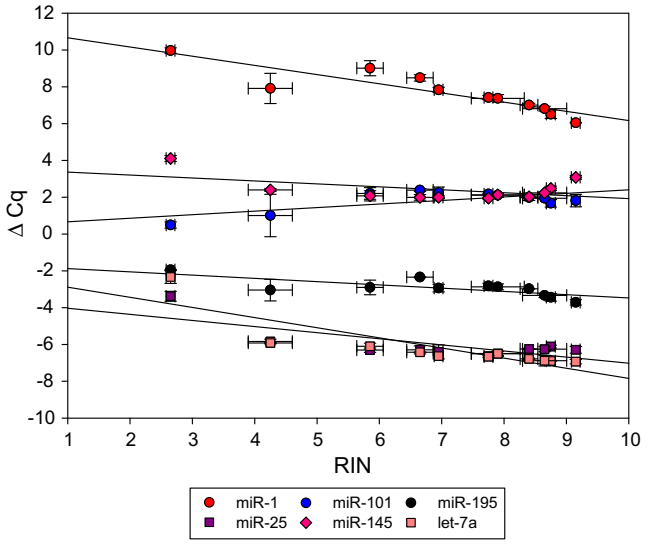

Fig. 5. miRNA expression data for WBC normalized with an RG index according to the ΔCq model.

The positive impact of normalization on biased expression data was not as effective in miRNA as in mRNA. Despite normalization, for almost all results a statistically significant correlation between the RIN and the ΔCq value occurred.

4. Discussion

It is generally accepted that sustaining of high RNA quality is one of the keys to get reliable and reproducible results from mRNA expression analysis [2,12]. This finding should be kept in mind for new applications also dealing with nucleic acids, e.g. expression profiling of miRNAs. Interestingly, samples with low total RNA quality showed the highest concentrations of miRNA. These data suggest an impairing influence of total RNA also for miRNA quantification and raised the question, if these results show a biological phenomenon or are due to a technical bias. Thus, in the actual study the influence of total RNA integrity on miRNA quantification and expression analysis was to be investigated.

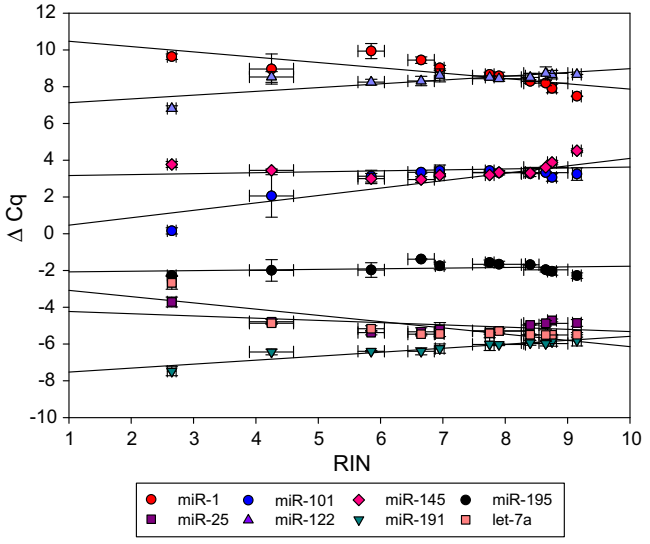

Fig. 6. miRNA expression data for WBC normalized with the mean expression value according to the ΔCq model.

As expected, a significant rise in miRNA amount occured with ongoing RNA degradation. This is caused by the formation of small RNA fragments by degradation of longer RNAs. Due to their length, these small fragments could reach the analytical range analyzed by the small RNA lab-on-chip assay and therefore lead to an overestimation of the miRNA amount. Hence, the concentration measurement using the 2100 Bioanalyzer is just reliable for RNA samples with good RNA quality. In consequence, it is recommended to consider the miRNA not as an isolated solitaire fraction, but always in combination with the mRNA and total RNA. Also, the definition of the miRNA fraction on the applied small RNA assay is questionable. Herein, all fragments with a length of 15–40 nt are defined as miRNAs, although in literature miRNAs are considered to have a length of 18–25 nt [16]. Thereby, it may be expected that even in samples with a low degree of fragmentation undefined RNA fragments are accounted as miRNAs and the miRNA amount may be overestimated even in samples with good RNA integrity. These results show clearly that it is hardly possible to quantify exactly the real amount of miRNA in a biological sample with existing methods. Coming technical innovations may give the possibility to solve these complex problems.

For gene expression analysis, mRNA as well as miRNA profiles have been investigated in this study to get a whole view over gene expression and to verify former results. A highly significant, negative correlation between the RIN and the Cq value for mRNA could be observed. Therefore, the results of earlier studies of Fleige and coworkers could be clearly confirmed [1,2]. Similar to mRNA, there is also a highly significant, negative correlation between the RIN and the Cq value for miRNA. From the lower slope of the regression line (1.578 vs. 0.784) it could be inferred that the compromising effect is less pronounced in comparison to mRNA. Due to their length miRNAs seem to be more stable and exhibit less recognition sites for nucleases. We conclude that miRNAs might be less affected by the overall degradation of total RNA compared to longer mRNAs.

To deal with factors adversely affecting the performance of RT-qPCR, it is important to apply a suitable normalization strategy in data analysis. The normalization of expression data can partly reduce the impairing influence of RNA quality on the performance of RT-qPCR [15]. For mRNA, the use of an RG index calculated as the geometric mean of multiple RG is generally accepted [15]. By now, there is no universally valid guideline for normalization of miRNA expression data. In literature, different strategies are described. Other endogenous small RNAs (nuclear and nucleolar RNAs) are used as internal control and also universal "reference miRNAs" (miR-17-5p, miR-103, miR-191) have been described [17]. miR-191 is also defined as a proper normalizer by GenEx in the current study, but just for one tissue. This finding suggests that normalizers for miRNAs are tissue and species specific just like normalizers for mRNA studies. They should not be determined generally, but tested for each experiment separately. Recently published data proved that normalization using the mean expression value or stable endogenous miRNAs used similarly to the RG index showed the best reduction of technical variances in RT-qPCR data [18]. The mean expression value is mostly used for high-throughput experiments like microarrays or qPCR array setups, whereas an RG index serves as normalizer in experiments focusing on limited number of genes like RT-qPCR. Both methods have been tested for normalization of the expression data from the current study. Looking at the genes investigated in the actual experiment, the normalization with the mean expression value gave comparable results to the normalization using RG. For the actual study, we could show that the normalization using the mean expression value may also be practicable for experiments with a limited number of genes and its application seems not just to be valid for profiling of large numbers of genes.

Obviously, the normalization of miRNA is much more sensitive to RNA quality than the normalization of mRNA. In spite of normalization, for almost all measured miRNAs and all tissues a significant correlation of the RIN and the ΔCq remained. This result was comparable for normalization with an RG index or either the mean expression value. It is evident that the linearity is just interrupted by outliers in the range of very low RNA quality (degradation step 10/11). Thus, the threshold of a RIN = 5 for gaining reliable PCR results, which was stated by Fleige et al. [1] could be confirmed for mRNA and also stated for miRNA by the actual study.

In conclusion, it could be shown that mRNA and miRNA quantification using the lab-on-chip technology is influenced by the overall total RNA integrity. Ongoing RNA degradation is accompanied by the formation of small RNA fragments leading to an overestimation of the miRNA amount. Thus, miRNA should not be considered as a solitaire fraction, but always as a part of the entire total RNA in respect to mRNA and total RNA quality. Using a small RNA assay is not an optimal method for exact quantification of the real miRNA amount. This assay is exceedingly influenced by the total RNA integrity and only reliable for samples with good RNA quality. Furthermore, the definition of the miRNA fraction in the entire small RNA fraction is questionable. The performance of RT-qPCR is impaired by decreasing RNA quality for miRNA similar to mRNA, but to a lower magnitude. The application of an appropriate normalization method can partly reduce the comprising degradation problem in RT-qPCR.

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Appendix II

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Effect of trenbolone acetate plus estradiol on transcriptional regulation of metabolism pathways in bovine liver

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Abstract

Background: The use of anabolic steroids is forbidden for food producing animals in the EU. Owing to the advantages of anabolics for production profitability, illegal application is appealing. Anabolics are known to influence gene expression of several tissues. We focused on the liver because of its important role in nutrient and hormone metabolism. The aim of the present study was to find differentially regulated metabolic pathways, which might be used as treatment biomarkers.
Material and methods: A total of 18 Nguni heifers were allocated equally to a control group and a treatment group and were implanted with Revalor H. Expression of 34 target genes was measured using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).
Results: Upregulation of androgen receptor and insulin-like growth factor 1 (IGF-1) and downregulation of IGF-2, insulin-like growth factor binding protein 2, steroid hormone binding globulin, insulin receptor α, insulin receptor β, tyrosine aminotransferase, 17β-hydroxy steroid dehydrogenase 2,3-hydroxy-methylglutaryl-coenzym-A-synthase, cathepsin B, hepatocyte growth factor, steroidogenic acute regulatory protein, apolipoprotein 2 and tumor necrosis factor α was demonstrated.
Conclusion: Several biochemical pathways showed different regulations on mRNA level under the influence of trenbolone acetate plus estradiol. The inhibition of nutrient metabolism and protein breakdown seems to support growth processes. IGF-1 plays an important role in growth and development and thus the upregulation of IGF-1 could be responsible for the stimulation of growth in treated animals. The upregulation of IGF-1 could also be revealed as a possible risk factor for the generation of artherosclerotic plaques, which are known as long-term side effects following the use of anabolic steroids. Principal components analysis of RT-qPCR results showed that both groups arrange together and can be clearly separated. Therefore, these might be used as possible biomarkers in bovine liver.

Keywords: anabolic steroids; estradiol; liver; mRNA gene expression; principal components analysis; trenbolone acetate; reverse transcriptase quantitative polymerase chain reaction.

Introduction

Anabolic agents are used in animal husbandry owing to their capacity to increase growth rate, the amount of lean body mass and feed efficiency (1). According to the profitability in lean meat production, the application of these drugs is admitted and wide spread in countries such as the USA and Canada. In the European Union (EU), the use of anabolic agents is only permitted for zootechnical purposes such as estrus synchronization and estrus induction, but is forbidden in food production since 1988 because the potential health risks for the consumer can hardly be estimated. Steroid hormones are known to influence many primary and secondary dependent tissues in the organism. Liver plays an important role in the metabolism of steroid hormones, e.g., hormone decomposition, and production of cholesterol as a precursor of steroid hormones. Additionally, many metabolic pathways in liver are controlled by steroid hormone action. Therefore, the liver has been chosen to be the main objective in this investigation. For this study, target genes participating in these pathways were identified and analyzed to reveal changes due to anabolic action. Target genes have been assigned to different functional groups.

After binding to intracellular receptors, reproductive hormones act indirectly on the muscle cell by stimulation of growth factors in the liver and several other organs (2). This observation provides the basis for the selection of different hormone receptors such as the androgen receptor (AR), estrogen receptors (ERα, ERβ), growth hormone receptor (GHR), insulin receptors (IRα, IRβ) and insulin-like growth factor 1 receptor (IGF-1R), as well as growth factors such as insulin-like growth factors (IGF-1, IGF-2) and insulin-like growth factor binding proteins (IGFBP-1, IGFBP-2). In addition, steroid hormone binding globulin (SHBG), which is responsible for the humoral transport of steroid hormones, has been investigated (3).

The rate of muscle growth is affected by variation of the rate of protein synthesis, the rate of protein degradation or both. This can be controlled by various hormones, e.g., estro-

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gens and androgens (4). To study the influence of anabolic hormones on protein metabolism, the catabolic enzymes cathepsin L (CTSL), cathepsin B (CTSB) and tyrosine aminotransferase (TAT) have been investigated.

The decrease in protein synthesis appears to be mediated in part by proinflammatory cytokines, such as tumor necrosis factor α (TNFα). Its suppression of the IGF-1 functional complex would mediate their catabolic effects (5–9).

The liver is the center of steroid hormone metabolism. Enzymes, which are involved in the biological inactivation of steroid hormones, could be influenced by the exogenous application of anabolic steroids. Therefore, different 17β-hydroxy steroid dehydrogenases (HSD17B2, HSD17B4, HSD17B8) have been examined (10, 11). In addition to steroid hormone elimination, the liver also plays an important role in steroidogenesis as the starting product cholesterol is synthesized in the liver. Thus, the following factors affecting cholesterol metabolism were investigated: 3-hydroxy-methyl-glutaryl-coenzym-A (HMGCoA-) synthase, HMGCoA-reductase and farnesyldiphosphate-farnesyltransferase (FDFT), which are key enzymes in the synthetic pathway of cholesterol formation from acetyl-CoA. Particular focus should be brought to the expression of FDFT as it catalyzes the first reaction only leading to cholesterol (12). Sterol regulatory element binding proteins (SREBP-1, SREBP-2) and their activator SREBP cleavage activating protein (SCAP) are important factors in the feedback regulation of cholesterol synthesis. The rate-limiting step in steroidogenesis is the transport of cholesterol from the outer to the inner mitochondrial membrane. This process is regulated by steroidogenic acute regulatory protein (STAR) (13, 14). The first reaction in the synthesis of steroid hormones is the conversion of cholesterol to pregnenolone. This reaction is catalyzed by cytochrome P450 11A1 (CYP11A1).

In humans, misuse of anabolics often leads to severe cardiac incidents due to atherosclerosis. Anabolic steroids are known to have an atherogenic influence by decreasing the levels of high-density lipoprotein (HDL)-cholesterol. This impairment of the plasma lipid level is associated with an increased risk for cardiovascular diseases (15). The drop-down in the HDL-fraction possibly originates from an accelerated HDL-catabolism, which is mediated by hepatic lipase (LIPC) and lipoprotein lipase (LPL) (16, 17). Different apolipoproteins (APOA1, APOC2) are also associated with an increased risk of atherosclerosis.

Sex hormones have been implicated in the generation of certain forms of cancer and liver tumors (18, 19). The protooncogenes myelocytomatosis cellular oncogene homolog (c-myc) and myeloblastosis viral oncogene homolog (v-myb) were analyzed. Carcinogenesis is usually linked with a dysregulation of cell proliferation. Thus, a member of one class of transcription factors, namely CAAT enhancer binding proteins (CEBPA), and the proliferation factor hepatocyte growth factor (HGF), which have an impact on the regulation of liver cell proliferation, were investigated.

Revalor H was chosen for treatment in the present study because it is an anabolic supplement widely used and licensed in the Republic of South Africa. Several investigations showed its efficacy in cattle and the beneficial effect on meat quality and quantity (20). In addition, the activity of this combination on day 42 has been proven, as the intended effect is persistent up to day 105 after implantation and residues of the applied drug are detectable in blood up to day 90 (21).

The aim of the present study was to identify transcriptional changes in metabolic pathways of liver, which could point to functional changes in liver physiology due to anabolic action. Additionally, it should be attempted to find gene expression patterns, which could be used as possible biomarkers for anabolic treatment. The application of appropriate biostatistical tools should help to extract the required information from the measured gene expression patterns (22).

Experimental

Experimental design

A total of 18 healthy non-pregnant, cyclic Nguni heifers were divided into a control group and a treatment group with nine animals in each group. Animals were housed under controlled conditions in a fenced loose barn without any access of unauthorized individuals. The treatment group was implanted with Revalor H, an anabolic preparation licensed in South Africa, containing 140 mg trenbolone acetate plus 20 mg estradiol-17β (Intervet, Spartan, South Africa) into the middle third of the pinna of the ear, according to the manufacturer's instructions.

Animal attendance was done by the Onderstepoort Veterinary Institute (Onderstepoort, Pretoria South Africa). The animals were housed and fed according to practice.

At slaughter on day 42, liver samples were collected. The tissue was conserved in RNA later (Applied Biosystems, Darmstadt, Germany), immediately after the removal and stored until analysis at −80°C.

Determination of progesterone values

Plasma samples for analysis of progesterone were taken during the course of the animal trial on days −11 and −5 before treatment and on days 2, 7, 9, 16, 22, 24, 29, 32 and 39 after the application of the anabolic preparation. Plasma progesterone levels were determined using an enzyme immunoassay as previously described (23).

Extraction of total RNA

Total RNA was extracted with the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Total RNA concentration was measured using the NanoDrop (peqLab Biotechnologie GmbH, Erlangen, Germany). $OD_{260/230}$ and $OD_{260/280}$ ratios were checked considering sample purity.

Analysis of RNA integrity

Control of RNA integrity was performed with the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The eukaryotic total RNA Nano Assay (Agilent Technologies) was used for sample analysis and the RNA integrity number (RIN) served as RNA integrity parameter. The RIN is calculated based on a numbering system from 1 to 10, with 1 being most degraded and 10 being most intact (24).

Primer design

Primer pairs were newly designed using published bovine nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or previously established primer sequences were used. Newly designed primers were ordered and synthesized at Eurofins MWG (Ebersberg, Germany).

Primer sequences of the reference genes (RGs) and the target genes are summarized in Table 1.

Reverse transcription (RT)

RNA samples were converted to cDNA using M-MLVH$^-$ reverse transcriptase (Promega, Regensburg, Germany). In total, 500 ng RNA was diluted to a final volume of 13 μL. The mastermix for the reverse transcription was prepared as follows: 4 μL 5× reaction buffer (Promega), 1 μL random primers (Invitrogen, Karlsruhe, Germany), 1 μL dNTP (Fermentas, St. Leon-Rot, Germany), 1 μL M-MLVH$^-$ reverse transcriptase (Promega). After adding 7 μL of the mastermix to the diluted sample, the plate was inserted into the Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany) and temperature protocol was started: 21°C, 10 min; 48°C, 50 min; 90°C, 2 min; 4°C hold. After cDNA synthesis, all samples were diluted 1:1 to a final volume of 60 μL. Reverse transcription was done in replicates and twice for the same biological sample and a pool of both reactions was created for every sample to gain a sufficient volume for analysis.

Quantitative PCR (qPCR)

Quantitative PCR was performed using MESA Green qPCR MasterMix plus for SYBR Assay w/fluorescein (Eurogentec, Cologne, Germany) by a standard protocol recommended by the manufacturer. All components necessary for real-time RT-qPCR were mixed in the reaction wells. The mastermix was prepared as follows: 7.5 μL 2× MESA Green qPCR MasterMix, 1.5 μL forward primer (10 pmol/μL), 1.5 μL reverse primer (10 pmol/μL), 3.0 μL RNase free water (5Prime, Hamburg, Germany). Per well, 13.5 μL mastermix plus 1.5 μL containing 12.5 ng cDNA was added. The plate was sealed, placed in the iQ5 Cycler (Bio-Rad, München, Germany), and the following PCR protocol was started: denaturation step (95°C, 5 min), cycling program (95°C, 3 s; annealing temperature according to Table 1, 60 s) and melting curve analysis.

Data evaluation

Expression data were analyzed using relative quantification. Suitable RGs for normalization of gene expression data were evaluated using the geNorm and Normfinder algorithm in GenEx v. 5.0.2.8 software (multiD Analyses AB, Gothenburg, Sweden). Optimal number of RGs was selected using pairwise variation analysis integrated in geNorm algorithm implemented in GenEx. The geometric mean of two selected and validated RGs (Ubiquitin, Histon H3) was used as reference gene index. Data were normalized and relatively compared to the control group according to the ΔCq model (25) with the following formulas:

$$\Delta Cq = Cq_{(\text{target gene})} - Cq_{(\text{reference gene index})}$$
$$\Delta\Delta Cq_{(\text{treatment})} = \Delta Cq_{(\text{treatment})} - \text{mean } \Delta Cq_{(\text{control})}$$

The expression ratio of an RG compared to the control group is expected to be $2^{-\Delta\Delta Cq}$.

Relative expression data were statistically evaluated using Sigma Stat 3.0 (SPSS Inc., Chicago, IL, USA). The Student t-test was used to determine p-values. Results with $p < 0.05$ were considered as statistically significant.

To visualize the multivariate response of the selected classifier genes to the treatment, the principal components analysis (PCA) method was employed using GenEx v. 5.0.2.8 (multiD Analyses AB). PCA involves a mathematical procedure that transforms a number of variables (here normalized expression values) into a smaller number of uncorrelated variables called principal components. By this, the dimensionality of the data is reduced to a number of dimensions that can be plotted in a scatter plot, here two dimensions. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible (26).

Results

Plasma progesterone levels

Analysis of plasma progesterone levels (Figure 1) showed a decrease in the treatment group compared to the control group. Significant differences between the control and treatment group could be observed at days 2, 22, 24, 29, 32 and 39 showing a clear initial response to the anabolic treatment and documenting the ongoing effectiveness of the anabolic preparation Revalor H until the end of the animal trial.

RNA integrity

All 18 samples were examined using the Agilent 2100 Bioanalyzer. The RNA samples showed good RNA quality with a mean RIN of 8.34 ± 0.36, which is perfectly suitable for PCR analysis (27).

Gene expression analysis

Out of 34 investigated genes, 11 were significantly regulated in the treatment group compared to the control group, four genes did not reach the significance level, but a trend for regulation could be observed ($p < 0.1$). Significant regulations could be obtained for genes from the receptors and endocrine factors group, the cholesterol metabolism group, the hormone decomposition and the protein metabolism group. The x-fold regulations of the significantly regulated genes are shown in Figures 2 and 3 as whisker-box plots. The box area of the plot represents 50% of all observations between the lower and upper quartile, the dotted line shows the sample median and whisker the outer 50% of the data.

In the endocrine group, an upregulation of AR and IGF-1 and a downregulation of IGF-2, IGFBP-2, SHBG, IRα and IRβ was observed. AR was upregulated in the treatment group in comparison to the control group by a mean factor of 1.68 ($p = 0.01$). IGF-1 was upregulated by a mean factor of 1.89 ($p = 0.012$). IGF-2 was downregulated by a mean factor of 0.77 ($p = 0.013$). IGFBP-2 showed a significant

Table 1 Sequences of the used primers with product length [bp], annealing temperature (T_M) and accession number, primers without accession numbers have been obtained from coworkers.

Functional group	Gene	Sequence [5′→3′]		Product length [bp]	T_M [°C]	Accession no.
Receptors	AR	FOR	CCT GGT TTT CAA TGA GTA CCG CAT G	172	60	AY862875
		REV	TTG ATT TTT CAG CCC ATC CAC TGG A			
	ERα	FOR	AGG GAA GCT CCT ATT TGC TCC	233	60	NM_001001443
		REV	GGT GGA TGT GGT CCT TCT C			
	ERβ	FOR	AAT CCA TCC TAC CCC TGG AG	164	64	NM_174051
		REV	ATT TGG GCT TGT GGT CTG TCG A			
	GHR	FOR	CCA GTT TCC ATG GTT CTT AAT TAT	137	60	NM_176608
		REV	TTC CTT TAA TCT TTG GAA CTG G			
	IRα	FOR	TCC TCA AGG AGC TGG AGG AGT	89	62	AJ488553
		REV	TTT CCT CGA AGG CCT GGG GAT			
	IRβ	FOR	TCC TCA AGG AGC TGG AGG AGT	111	60	AJ320235
		REV	TAG CGT CCT CGG CAA CAG G			
	IGF-1R	FOR	CCC AAA ACC GAA GCT GAG AAG	200	60	XM_606794
		REV	TCC GGG TCT GTG ATG TTG TAG			
Endocrine factors	IGF-1	FOR	CAT CCT CCT CGC ATC TCT TC	239	62	NM_00177828
		REV	CTC CAG CCT CCT CAG ATC AC			
	IGF-2	FOR	ACC CTC CAG TTT GTC TGT GG	166	62	BC126514
		REV	ACA CAT CCC TCT CGG ACT TG			
	IGFBP1	FOR	ACC AGC CCA GAG AAT GTG TC	244	64	NM_174554
		REV	GTT TGT CTC CTG CCT TCT GC			
	IGFBP2	FOR	AGC ATG GCC TGT ACA ACC TC	157	60	NM_174555
		REV	CCC TGC TGC TCG TTG TAG AA			
	SHBG	FOR	ACT TGG GAT CCA GAG GGA GT	188	62	NM_174555
		REV	TCC CCA TGG ATC TTC ACT TCC			
Protein metabolism	CTSB	FOR	GGT TGC AGA CCG TAC TCC AT	391	62	NM_174031
		REV	CAT TGT CAC CCC AAT CAG TG			
	CTSL	FOR	CAC TGG TGC TCT TGA AGG ACA	177	62	NM_174032
		REV	TAA GAT TCC TCT GAG TCC AGG C			
	TAT	FOR	ACC CTT GTG GGT CAG TGT TC	165	60	NM_001034590
		REV	ACA GGA TGG GGA CTT TGC TG			
Transcription factors/ proliferation	c-myc	FOR	TCT TGC GCC TAA ATT GAC CTA TTG	153	60	NM_001046074
		REV	GCC AAG GTT GTG AGG TTG TTC			
	v-myb	FOR	CTG TCA AGT CCA CCC CAG TTA	186	63	NM_176635
		REV	TTC TGA GGA TGG TTG GTG GAG			
	HGF	FOR	GAT GTC CAT GGG AGA GGA GA	170	62	NM_001031751
		REV	TCA GGA ATT GTG CAC CCA TAA TTA G			
	CEBPA	FOR	CCA AGA AGT CCG TGG ACA AG	184	62	NM_176784
		REV	AGT TCG CGG CTC AGT TGT TC			
Immune factors	TGFβ	FOR	ACG TCA CTG GAG TG TGC GG	155	63	NM_001166068.1
		REV	TTC ACG CCG TGA ATG GTG GCG			
	TNFα	FOR	TCT GCC ATC AAG AGC CCT TGC C	185	64	NM_173966
		REV	GCG ATG ATC CCA AAG TAG ACC TGC C			
Cholesterol metabolism	HMGCoA-Synthase	FOR	GAT GGT CGC TAT GCA CTG GT	132	60	BC112666.1
		REV	GCC CTC TCT CGA GGA CCA GA			
	HMGCoA-Reductase	FOR	CTC TCT AAA ATG ATC AGC AT	246	60	
		REV	TCA ACT TTT CTT TCT CTG TTT			
	FDFT	FOR	GAA ATG CGC CAT GCA GTA	198	60	
		REV	GGA GAT CGT TGG GAA GTC CT			
	SREBP-1	FOR	CCA GCT GAC AGC TCC ATT GA	67	60	
		REV	TGC GCG CCA CAA GGA			
	SREBP-2	FOR	CAG GTC CTG GTA CAG CCT CA	158	60	
		REV	GCT CTT ACC GGA ACT TGC AG			
	SCAP	FOR	GGT CAC TTT CCG GGA TGG	179	60	NM_001101889.1
		REV	TGG GTA GCA GCA GGC TAA GA			
Steroid metabolism	STAR	FOR	TGG AAA AGA CAC GGT CAT CA	154	63	NM_174189
		REV	CTG GGG CAT CTC CTC ATA GA			

(Table 1 continued)

Functional group	Gene	Sequence [5′→3′]		Product length [bp]	T_M [°C]	Accession no.
	CYP11A1	FOR	CGG AAA GTT TGT AGG GGA CAT C	177	63	NM_176644
		REV	ACG TTG AGC AGA GGG ACA CT			
	HSD17B2	FOR	CAT CTC AGG CAC GAG TCA AAT G	160	62	NM_001075726
		REV	CAC TGG GGA GAT GTC TGG ATC			
	HSD17B4	FOR	CGG ATG ACC CAA AGC ATT TTG C	176	62	NM_001007809.1
		REV	TCT GTC TCA CAA GGG CTC CAA			
	HSD17B8	FOR	GGG CAT CAC CAG AGA TGA AT	228	62	NM_001046324
		REV	CAA TCA CTC CAG CCT TGG AT			
Lipid metabolism	APOA1	FOR	TTT GGG AAA ACA GCT CAA CCT GAA	215	63	NM_174242
		REV	GCC ACT TCT TCT GGA ACT CG			
	APOC2	FOR	GGG TTT CTC ATC CTC CTG GT	240	63	NM_001102380
		REV	AAT CCC TGC ATA GGT GGT CAC			
	LIPC	FOR	CGC CAT TCA CAC CTT TAC CT	186	62	NM_001035410
		REV	TCA TGG GCA CAT TTG ACA GT			
	LPL	FOR	CTC CTG ATG ATG CGG ATT TTG TA	196	62	NM_001075120
		REV	ACC AGC TGA TCC ACA TCT CC			
Reference genes	H3	FOR	ACT CGC TAC AAA AGC CGC TC	232	60	NM_001014389
		REV	ACT TGC CTC CTG CAA AGC AC			
	UBQ	FOR	AGA TCC AGG ATA AGG AAG GCA T	426	60	NM_174133
		REV	GCT CCA CCT CCA GGG TGA T			

downregulation ($p = 0.001$) in the treatment group compared to the control group by 0.27. A downregulation by a mean factor of 0.66 was also observed in SHBG ($p = 0.0002$). IRα and IRβ were significantly downregulated ($p_{IR\alpha} = 0.02$; $p_{IR\beta} = 0.011$) by a mean factor of 0.77 and 0.69, respectively.

One gene in the steroid hormone decomposition group showed a significant regulation. HSD17B2 was downregulated by a mean factor of 0.73 ($p = 0.039$). STAR was not significantly regulated ($p = 0.06$) but showed a trend for downregulation by a mean factor of 0.64.

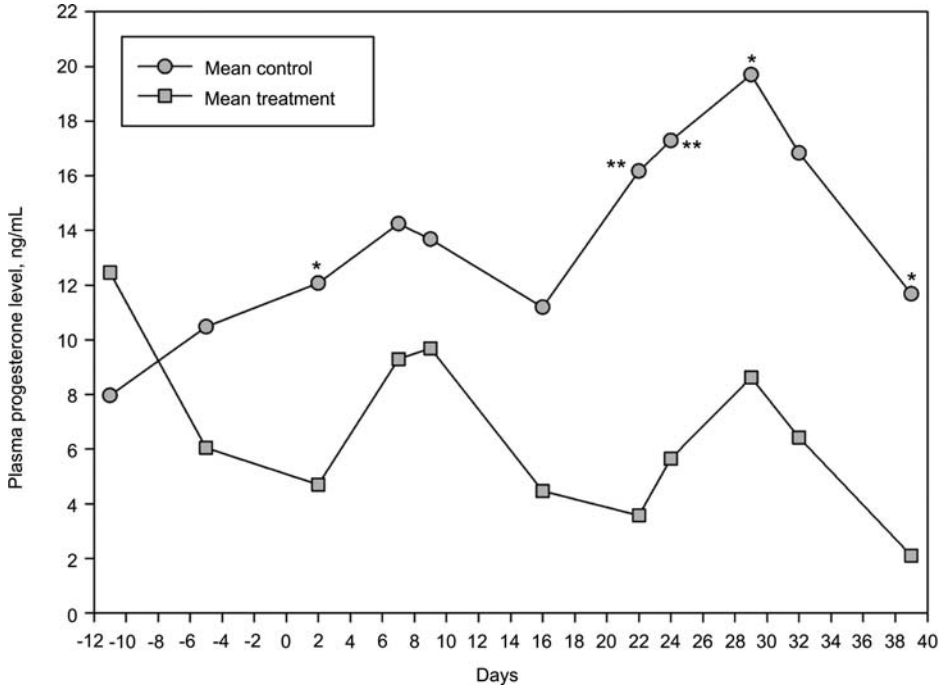

Figure 1 Plasma progesterone levels determined during the course of the study, showing significant differences between the control group and the treatment group at day 2, 22, 24, 29 and 39. *$p \leq 0.05$, **$p \leq 0.01$.

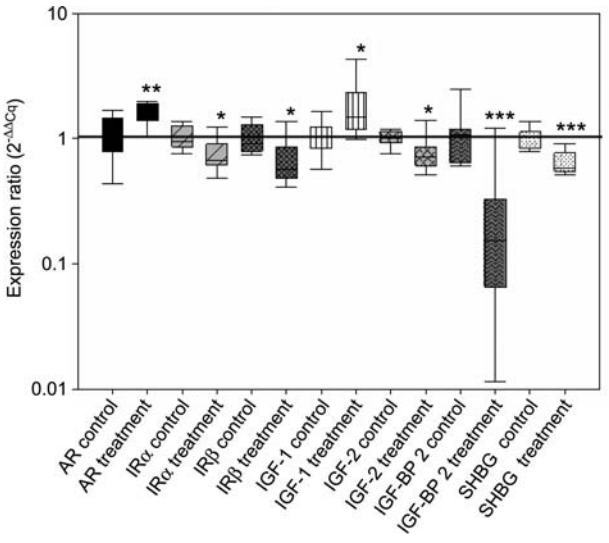

Figure 2 Expression ratio of regulated target genes under hormone treatment. *p < 0.05, **p < 0.01, ***p < 0.001.

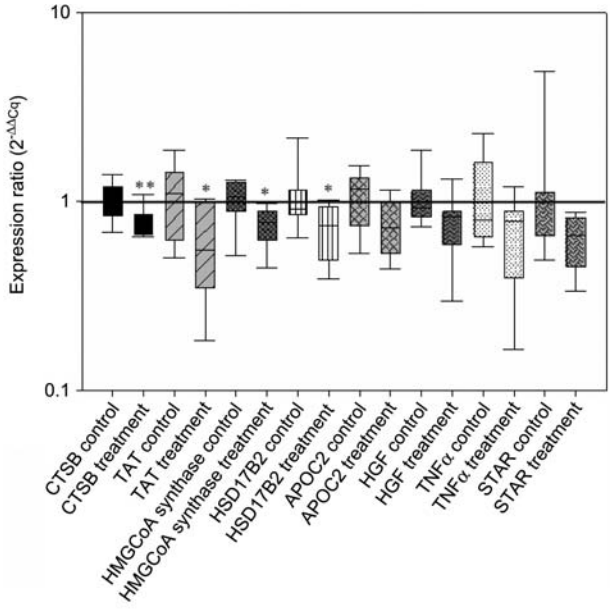

Figure 3 Expression ratio of regulated target genes under hormone treatment. *p < 0.05, **p < 0.01.

In addition, one gene in the cholesterol metabolism group was significantly regulated. For HMGCoA-synthase, a downregulation by a mean factor of 0.74 was observed (p = 0.023). APOC2 showed a trend for downregulation by a mean factor of 0.75, but this difference was not significant (p = 0.059).

In the protein and amino acid group, significant downregulations were shown for CTSB and TAT. CTSB was downregulated (p = 0.009) in the treatment group in comparison to the control group by a mean factor of 0.77. TAT was downregulated (p = 0.028) by a mean factor of 0.61. For TNFα, no statistically different expression could be determined, but a trend for downregulation by a mean factor of 0.70 was visible (p = 0.082).

The proliferative factor HGF showed a trend for downregulation by a mean factor of 0.78, but the difference was not significant (p = 0.07).

PCA was produced as shown in Figure 4 by plotting the expression results of regulated genes of all samples of both groups by their first end second principal component. Gray crosses represent samples of the control group, black triangles display the samples of the treatment group. A distinct control group could be seen separated from the treatment group, showing that there was a multitranscriptional response to the treatment.

Discussion

Steroid hormones are known to alter expression of various genes by acting as transcription factors and thereby mediate their physiological effect. In the current study, the influence of the anabolic combination trenbolone acetate plus estradiol on gene expression in liver of Nguni heifers was investigated. It is generally known that the effect of the anabolic combination Revalor H is consistent during an experimental period as in the present trial (21) and also the actual measurement of the plasma progesterone levels showed a significant difference between the control and the treatment with the most distinct differences at days 22–39 proving the effectiveness of Revalor H until the end of the experimental course. Progesterone levels were lower in the treatment group compared to the control group due to negative feedback mechanisms following the application of exogenous hormones. Therefore, it can be assumed that gene expression changes are due to the anabolic treatment.

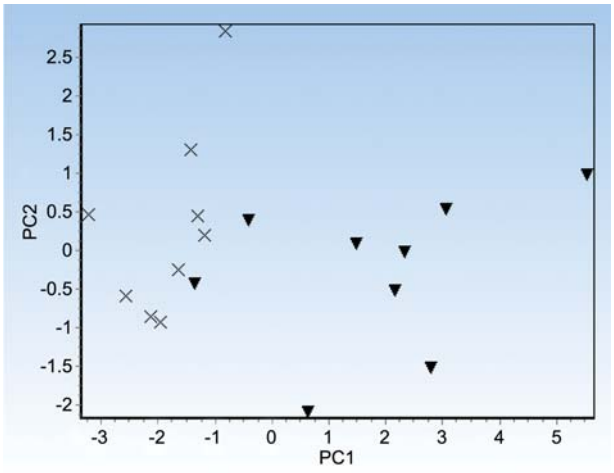

Figure 4 PCA of regulated target genes with control animals represented by grey crosses and treated animals represented by black triangles.

The actual analysis showed an upregulation of IGF-1. This is in agreement with the well-established hypothesis that steroid hormones increase IGF-1 gene expression and also with investigations of other groups, which showed an upregulation for IGF-1 in feedlot cattle and rats (28–30). Several other publications (30–32) showed in the case of an upregulation of IGF-1 mRNA also increased concentrations of circulating IGF-1 protein levels in lambs and steers, thus it can be assumed that regulation of IGF-1 levels really occurs on the level of transcription by directly affecting the IGF-1 gene. A higher synthesis of IGF-1 in the liver, which is the main source for IGF-1, could be partially responsible for the higher serum levels (32). Owing to the para- plus endocrine action of IGF-1, it can be assumed that the growth promoting effect of anabolics on muscle tissue is mediated by circulating IGF-1 produced in liver.

In addition to skeletal muscles, IGF and IGFBP are also known to have an influence on smooth muscles. IGF-1 and IGF-2 have been shown to stimulate the migration and proliferation of smooth muscle cells, whereas IGF-1 is more potent in the postnatal phase than IGF-2 in the prenatal phase (33, 34). This response can be modulated by binding of IGF to IGFBP. In particular, IGFBP-2 can completely suppress the effect of IGF-1. The migration of smooth muscle cells is one of the initial events in the formation of atherosclerosis, which is the major reason for cardiovascular side effects after misuse of anabolic steroids in humans (16). In the present study, an upregulation of IGF-1 and a downregulation of IGFBP-2 was recognized. This observation could be a sign of an augmented migration of smooth muscle cells. These changes on the gene expression level and the known disturbed lipid profile (35) could cause the increased risk of atherosclerosis following abuse of anabolics.

The endocrine factor SHBG contributes to the humoral transport of steroid hormones and is mainly synthesized in the liver. The expression of this factor has never been examined in correlation with the action of anabolic steroids, neither in human nor in bovine, but the literature describes a decreased protein level of SHBG in human users of anabolic steroids (3). In calves, the potential of SHBG binding level to be used as a biomarker for anabolic treatment has also been tested (36). These findings are in line with our results of a downregulation of SHBG mRNA in liver. During the exogenous application of anabolic steroids, the organism could reduce the synthesis of SHBG on the mRNA level to restrict the transport of steroid hormones and thereby facilitate degradation of unbound steroids.

The biosynthesis of steroid hormones requires cholesterol and its biosynthesis is in part controlled by genetic expression of the enzymes HMGCoA-Synthase, HMGCoA-Reductase and FDFT. In our experiment, HMGCoA-Synthase was downregulated; but owing to the fact that we could not detect any regulation for HMGCoA-Reductase or FDFT, it is difficult to make any statement concerning the influence of a treatment with trenbolone acetate plus estradiol on cholesterol biosynthesis.

Cholesterol is the initial component in the synthesis of steroid hormones, thus its synthesis should be reduced with the application of exogenous hormones. After its synthesis, cholesterol is transported to the inner mitochondrial membrane for the generation of bile acids or steroid hormones. This transfer is mediated by STAR and is thereby the first step in steroid hormone generation (14). STAR showed a trend for downregulation in the current study. This leads to the hypothesis that steroid hormone synthesis is blocked under the influence of exogenous hormones. However, CYP11A1, the enzyme catalyzing the first and rate-limiting step in steroid syntheses from pregnenolone to progesterone showed no significant regulation. Thus, this block could be introduced prior to the actual pathway of steroid synthesis at the level of formation and processing of the initial product, which is visible in the downregulation of HMGCoA-Sythase and STAR.

HSD17B2 belongs to the group of oxidative steroid dehydrogenases, which direct their activity towards the inactivation of steroid hormones. HSD17B2 itself catalyzes the reaction from estradiol to estrone and testosterone to Δ4-androstendione (11). The finding of a downregulation of HSD17B2 in the current study was unexpected. This enzyme should be upregulated under the influence of exogenous hormones to accelerate the degradation of the applied substances. However, no precise information on physiological responses could be given based on gene expression results. So possibly, the inactivation of the applied hormones is regulated on another level.

The liver plays a pivotal role in glucose metabolism and homeostasis. Glucose uptake and storage as glycogen by hepatocytes is mediated by the interaction of insulin with hepatic insulin receptors. In the present study, IRα as well as IRβ were downregulated leading to an impaired insulin sensitivity of the liver. This mechanism could be responsible for the reduction of glycogen synthesis, lipogenesis and protein synthesis due to the action of anabolic steroids. Because these nutrients can be less metabolized by the liver, they are kept in the blood and are available for proliferation processes in the skeletal muscles.

This is consistent with the observed downregulation of TAT and CTSL. TAT is an enzyme that is mainly located in the liver catalyzing the first step in the degradation pathway of the amino acid tyrosine (37). The downregulations of the protease CTSB and the aminotransferase TAT could be responsible for an anti-catabolic effect and for protein plus nitrogen retention. Together with the upregulation of IGF-1, these effects could be responsible for muscle growth under the influence of anabolic steroids.

Unlike the natural androgen testosterone, which mediates its anabolic action by binding to the AR and also, after its conversion by an aromatase to estradiol, to ER (ERα and ERβ), the xenobiotic androgen trenbolone acetate is able to bind to AR, GR and progestin receptor (2). AR and GR as well as both ER subtypes were investigated in this study. As just an upregulation of AR occurred, it could be assumed that the anabolic effects of the applied treatment are mainly due to trenbolone acetate by its binding to AR. However, it should be noted that there are also other regulation levels of physiological function such as post-transciptional modifica-

tions, which can participate in the regulation of the mentioned receptors that could not be shown to be significantly regulated in this investigation. In addition to the regulation of gene transcription, steroid hormones are known to influence the stability and degradation of existing mRNA and especially to regulate the concentrations of their own receptors (38, 39). Thus, changes in gene expression on the transcriptional level of certain target genes could only be a part of changes in biochemical pathways.

In biomarker research, the application of valid bioinformatical and statistical tools for data processing is important to extract the required information. In most of the fields that biomarkers are searched for, it is general that not only one factor is used but a variety of factors are integrated to a biomarker pattern. Therefore, in particular methods for dimensionality reduction are needed to transform the high-dimensional data sets (22). In the present study, PCA has been applied as a multivariate analysis method, which can be used, if more than three components should be taken into account. The PCA of all regulated genes showed that the treatment differs from the control animals although single genes are distributed within similar ranges. It could be observed that both groups arrange together and that a difference between control and treatment groups can be monitored. In addition to discussed regulations of single genes, this illustration could help to reveal the transcriptional shift in the treatment group, which could be an indication for functional changes in liver physiology following anabolic treatment. Also, this observation is a first indication that it is possible to elaborate a gene expression pattern making it is possible to develop a screening method to control the misuse of anabolic hormones in cattle.

In conclusion, several biochemical pathways, which were differentially regulated on mRNA level under the influence of trenbolone acetate plus estradiol, have been identified. Most changes seem to support growth processes in the organism. The metabolism of nutrients such as glucose in the liver could be suppressed by the decrease of the insulin sensitivity mediated by the downregulation of IRα and IRβ. These nutrients are kept in the blood flow and could be available for skeletal muscle growth. An inhibition of the protein breakdown was observed. Also, IGF-1 could be responsible for the stimulation of growth in treated animals (28). Additionally, the upregulation of IGF-1 could also be revealed as a possible risk factor for the generation of artherosclerotic plaques, which are known long-term side effects following the use of anabolic steroids.

PCA of RT-qPCR results showed that both groups arrange separately and that a difference between control and treatment groups becomes obvious. Therefore, the measured gene expression pattern could possibly be used as biomarkers for anabolic treatment with trenbolone acetate plus estradiol in bovine liver.

Acknowledgements

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Appendix III

INFLUENCE OF ANABOLIC COMBINATIONS OF AN ANDROGEN PLUS AN ESTROGEN ON BIOCHEMICAL PATHWAYS IN BOVINE UTERINE ENDOMETRIUM AND OVARY

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ABSTRACT

The application of anabolic steroids in food producing animals is forbidden in the EU since 1988, but the abuse of such drugs is a potential problem. The existing test systems are based on known compounds and can be eluded by newly emerging substances. The examination of physiological effects of anabolic hormones on different tissues to indirectly detect misuse might overcome this problem.

Two studies were conducted with post-pubertal 24-months old Nguni heifers and pre-pubertal female 2-4 weeks old Holstein Friesian calves, respectively. The animals of the accordant treatment groups were administered combinations of estrogenic and adrogenic compounds. The measurement of the gene expression pattern was undertaken with RT-qPCR. Target genes of different functional groups (receptors, angiogenesis, steroid synthesis, proliferation, apoptosis, nutrient metabolism and others) have been quantified.

Several biochemical pathways were shown to be influenced by anabolic treatment. Both studies identified significant regulations in steroid and growth factor receptors (AR, ERβ, LHR, FSHR, Flt-1, PR, IGF-1R, Alk-6), angiogenic and tissue remodeling factors (VEGFs, FGFs, BMPs, ANGPT-2, MMPs, TIMP-2, CTSB), steroid synthesis (S5A1, HSD17, CYP19A1), proliferation (TNFα, IGF-1, IGFBPs, p53, c-fos; CEBPD, c-kit), apoptosis (CASP3, FasL, p53) and others (C7, INHA, STAR). Several genes were regulated to opposite directions in post-pubertal compared to pre-pubertal animals. PCA for Nguni heifers demonstrated a distict seperation between the control and the treatment group.

In conclusion, anabolics modify hormone sensitivity and steroid synthesis, and they induce proliferative effects in the whole reproductive tract (uterus and ovary) as well as anti-angiogenic effects in the ovary. However, the extent will depend on the developmental stage of the animals.

54 Key words: anabolic steroids, reproductive tract, mRNA gene expression, RT-qPCR, PCA

55

1. INTRODUCTION

The use of anabolic agents in animal husbandry is very profitable as treated animals are growing faster and exhibit a higher amount of lean meat due to nitrogen retention and a decrease in total body fat [1]. In some countries, e.g. USA and Canada, the utilisation of these substances is common and widely spread. In Europe, as the health risk for consumers caused by the hormone residues can hardly be estimated, the application of anabolic steroids is not admitted. Nevertheless, their misuse is a potential problem and new and sensitive detection methods have to be established to trace upcoming unknown compounds. One auspicious approach to indirectly uncover the illegal use of growth promoting substances might be by monitoring the different expression levels of genes participating in biochemical pathways, which are known to be influenced by anabolic steroids hormones. The actual study focused on two major reproductive organs (uterine endometrium and ovary) as these organs are primary hormone-dependent tissues. Addiotionally, few is known about the influences of anabolic steroids on these tissues [2] and the physiological changes induced by growth promoting substances have never been examined in bovine ovary by now. The following target genes have been examined in the actual work due to their involvement in metabolic processes regulated by steroid hormones.

The steroid hormone receptors androgen receptor (AR), estrogen receptors (ERα, ERβ), progestin receptor (PR), glucocorticoide receptor (GR) as well as growth hormone receptor (GHR) and IGF-1 receptor (IGF-1R), which are important for steroid hormone signaling, have been analyzed. Luteinizing hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR) were also investigated, as these are implicated in the negative feedback mechanism of reproductive hormones. The angiogenic vascular endothelial growth factors (VEGFs) and their receptors (FLK-1, Flt-1), fibroblast growth factors (FGF-1, -2, -7), thrombospondin (THBS), matrix metalloproteinases (MMP-2, MMP-23B) and their inhibitor (TIMP-2), and angiopoietins (ANGPT-1, ANGPT-2) have been analyzed because angiogenesis is a process naturally occuring in reproductive tissues and is regulated by steroid hormones [3;4]. For the

most part, steroid synthesis in the female organism takes place in ovary. This process is physiologically regulated by a negative feedback and though could be influenced by the application of exogenous reproductive hormones. Different key enzymes of steroids synthesis, namely Cytochrom P450 enzymes (CYP11A1, CYP19A1), hydroxysteroid-dehydrogenases (HSD3B1, HSD17B1, HSD17B3, HSD17B8, HSD17B11) and 5α-reductase (S5A1) have been exmined [5-7]. The application of anabolic steroids has been correlated to cancer [8]. The development of cancer is associated with the overexpression of so called proto-oncogenes, which affect the regulation of cell growth and differentiation. For this study, the tumorsuppressor gene p53 (p53) as well as the transcription factors c-fos, c-jun and c-myc, which are postulated as downstream targets of ER signaling, have been measured [9;10]. Due to the trophic effect of anabolics, apoptosis and proliferation are interesting functional pathways to investigate. Insulin-like growth factors (IGF-1, IGF-2) and their regulators IGFBP-2 and IGFBP-3 [11;12], growth differentiation factor 9 (GDF-9), bone morphogenetic factors (BMP-2, BMP-4, BMP-15) and the receptors mediating their action (Alk-5, -6, BMPRII) [13], transforming growth factor β (TGFβ), tumor necrosis factor α (TNFα), cyclin D2 (CYD2) [8], the negativ regulator Prohobitin (PHB) and the transcription factors CCAAT/enhancer binding protein β and δ (CEBPB; CEBPD) represent factors, which are implicated in proliferative events [10]. The apoptosis group consists of anti-apoptotic members of the Bcl-family (bcl-2, bcl-xl) as well as caspases (CASP3, CASP8), Fas receptor (FAS) and Fas ligand (FASL), which are important mediators of cell death [14]. Glycolysis is an important pathway occuring in every somatic cell. The degradation of glucose conduces to the supply of biological energy for different physiological processes. Hexokinase (HK) and lactatdehydrogenase (LDH), which are key enzymes of glycolysis, have been sorted to the energy metabolism group. Concerning the development of a higher proportion of lean meat in animals treated with anabolic steroids all genes associated with protein turnover are of note. Therefore, Cathepsin B (CTSB), cathepsin L (CTSL) and calpastatin (CAST) have been analyzed [15]. Several other factors, which are correlated to anabolic steroid homone action, like steroidogenic acute regulatory protein (STAR), Lactotransferrin (LTF), complement

components (C3, C7), cyclooxygenase 2 (COX-2), Smad2 and Inhibin A (INHA) were investigated [16-20]

Aim of the study was to find differentially regulated metabolic pathways in bovine reproductive tract and to compare the transcriptional response of post- and pre-pubertal animals. Using appropriate biostatistical methods, it should be attempted to find gene expression patterns, which could be used as possible biomarkers for anabolic treatment in cattle [21].

2. MATERIAL AND METHODS

2.1 Experimental design

In the first study ("South Africa study"), 18 healthy, post-pubertal, non-pregnant, 24-months old Nguni heifers were divided into a control and a treatment group of nine animals each. The animal attendance was done by the Onderstepoort Veterinary Institute (Onderstepoort, Pretoria South Africa). The treatment group was implanted with Revalor H (Intervet, Spartan, South Africa), an anabolic preparation licensed in South Africa and containing 140 mg trenbolone acetate (TBA) plus 20 mg estradiol-17ß (E2) according to the manufacturer´s instructions into the middle third of the pinna of the ear and was treated for 42 days until slaughter.

For the second animal study ("pour on anabolics study"), 20 pre-pubertal, female Holstein Friesian calves were randomly assigned to four groups of five animals each. Group 1 remained untreated and served as control. Animals of group 3 and group 4 were treated once or three times in weekly intervals, respectively, with a hormone mix containing 25 mg estradiol benzoate (Sigma Aldrich, Zwijndrecht, The Netherlands), 60 mg testosterone decanoate (Sigma Aldrich) and 60 mg testosterone cypionate (Sigma Aldrich). The hormone mix was applied in two different ways: per intra muscular injection (one animal per group) or via pour on treatment (four animals per group). For pour on treatment, animals were shaved on the back from neck to tail and 10 mL of the hormone mix were rubbed onto the skin. Four substances served as carrier solvents for the pour on treatment to ensure the transit of the hormone mix from the skin into the organism: Ivomec (Spruyt Hillen, IJsselstein, The Netherlands), dimethyl sulfoxide (DMSO) (Spruyt Hillen), Miglyol 840 (Spruyt Hillen) and diethylen glycol monobuthyl ether (DEGMBE) (Spruyt Hillen). For injection, Arachide oil (Spruyt Hillen) was used. Group 2 received only the carrier substances without the hormone mix three times in weekly intervals to serve as a carrier control group. Animals were slaughtered 92 days after the beginning of the experiment. Animal attendance was done

145 according to practice and the treatment protocol has been approved by the ethical committee
146 of the “Regierung von Oberbayern” (Upper Bavaria, Germany).
147 At slaughter, uterus and ovary samples were collected. Tissues were conserved in RNAlater
148 (Applied Biosystems, Darmstadt, Gemany) immediately after the removal and further stored
149 at -80°C.
150
151 *2.2 Extraction*
152 Samples were extracted with the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to
153 the manufacturer´s recommendations. RNA concentration was measured after extraction
154 using the NanoDrop (peqLab Biotechnologie GmbH, Erlangen, Germany). $OD_{260/230}$ and
155 $OD_{260/280}$ ratios were checked considering sample purity.
156
157 *2.3 Analysis of RNA integrity*
158 Intactness of sample material is crucial fin qPCr experiment [22], so control of RNA integrity
159 was performed with the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) using
160 Eukaryotic Total RNA Nano Assay (Agilent Technologies). The RNA integrity number (RIN)
161 served as RNA integrity parameter. The RIN is calculated based on a numbering system
162 from 1 to 10, with 1 being most degraded and 10 being most intact [23].
163
164 *2.4 Primer design*
165 Primer pairs (table 1) were either newly designed using published bovine nucleic acid
166 sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or previously
167 established primer sequences were used [2;24;25]. Newly designed primers were ordered
168 and synthesized at Eurofins MWG (Ebersberg, Germany).
169
170 *2.5 Reverse transcription*
171 Per sample, 500 ng (100 ng/µL) of total RNA have been converted to cDNA using M-MLV H^-
172 reverse transcriptase (Promega, Regensburg, Germany). The mastermix for the reverse

transcription was prepared as follows: 8 µL RNase free water (5Prime, Hamburg, Germany), 4 µL 5 x reaction buffer (Promega), 1 µL Random Primers (Invitrogen, Karlsruhe, Germany), 1 µL dNTPs (Fermentas, St. Leon-Rot, Germany), 1 µL M-MLV H^- reverse transcriptase. The reaction mix was inserted in the Eppendorf Gradient Mastercyler (Eppendorf, Hamburg, Germany) and the here stated temperature protocol was started: 21°C, 10 min; 48°C, 50 min; 90°C, 2 min; 4°C hold. cDNA samples were diluted with 40 µL of RNAse free water (5Prime) to a final volume of 60 µL. Reverse transcription was done in duplicates for every sample.

2.6 Quantitative PCR

Quantitative PCR (qPCR) was performed using MESA Green qPCR MasterMix plus for SYBR Assay w/ fluorescein (Eurogentec, Cologne, Germany) by a standard protocoll recommended by the manufacturer. The mastermix was prepared as follows: 7.5 µL 2 x MESA Green qPCR MasterMix; 1.5 µL forward Primer (10 pmol/µL); 1.5 µL reverse Primer (10 pmol/µL); 3.0 µL RNAse free water (5Prime). For a total volume of 15 µL, 1.5 µL cDNA were mixed with 13.5 µL of the mastermix in a 96-well plate. The plate was heat-sealed with the Eppendorf Heat-Sealer (Eppendorf), placed in the iQ5 Cycler (Bio-Rad, Munich, Germany) and the following PCR protocol was started: denaturation step (95°C, 5 min), cycling program [95°C, 3 s; primer specific annealing temperature (see table 1), 60 s] and melting curve analysis.

2.7 Data evaluation

Expression data were analysed using relative quantification. The geometric mean of three RG [Ubiquitin (UBQ), Histon 3 (H3), βActin (ACTB)] was used as reference index. Data were normalized and relatively compared to the control group according to the ΔΔCq-model with the following formulas [26]:

$$\Delta Cq = Cq_{(target\ gene)} - Cq_{(reference\ gene\ index)}$$

$$\Delta\Delta Cq = \Delta Cq_{(treatment\ group)} - mean\ \Delta Cq_{(control\ group)}$$

The expression ratio of the treatment group compared to the control group is expected as $2^{-\Delta\Delta Cq}$ and represents the x-fold regulation with a value of 1.00 indicating no expression change after treatment. Relative expression data were statistically evaluated using Sigma Stat 3.0 (SPSS Inc., Chicago, IL). The determined *p*-values of the statistical significance were examined using Students´s t-test. Results with $p \leq 0.05$ were considered as statistically significant, results with ≤ 0.1 were considered to show a statistical tendency. To visualize the multivariate response of the selected classifier genes to the treatment, the method of principle components analysis (PCA) was employed using GenEx v. 5.0.2.8 (multiD Analyses AB). PCA involves a mathematical procedure that transforms a number of variables (here normalized expression values) into a smaller number of uncorrelated variables called principal components. By this, the dimensionality of the data is reduced to a number of dimensions that can be plotted in a scatter plot, here two dimensions. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible [27].

3. RESULTS

3.1 RNA integrity

RNA integrity was controlled to prove the suitability of the sample material for RT-qPCR [22]. In the South Africa study, samples from uterine endometrium horn showed a mean RIN of 8.3 ± 0.49 (n=12), samples from uterine endometrium corpus showed a RIN of 7.9 ± 0.49 (n=12) and samples from ovary showed a mean RIN of 7.5 ± 0.98 (n=11). Ovary samples from the pour on study showed a mean RIN of 7.7 ± 0.70 (n=19). Therefore, all samples were suitable for RT-qPCR.

3.2 Gene expression results from post-pubertal Nguni heifers

In uterine endometrium corpus, significant regulations could be demonstrated in 7 genes (table 2). Up-regulations occurred for the receptor AR (p=0.012), the angiogenic factor VEGFA (p=0.044), the complement system factor C7 (p=0.022) and the proliferative factors c-fos (p=0.028) and CEBPD (p=0.024). A down-regulation could be shown for CASP3 (p=0.005) and BMP-4 (p=0.020). A trend for up-regulation could be obtained for FGF-7 (p=0.088) and IGFBP-3 (p=0.085), but these differences were not statistically significant.

Significant regulations for 8 genes could be detected in uterine endometrium horn (table 3). Up-regulations resulted for the receptor AR (p=0.014), the proliferative factors TNFα (p=0.018) and FasL (p=0.049), and the complement system factor C7 (p=0.001). Down-regulations arose for CASP3 (p=0.00001), CTSB (p=0.033) and BMP-4 (p=0.041). IGF-1 R (p=0.083), MMP-2 (p=0.057) and p53 (p=0.076) showed a trend for down-regulation, whereas a trend for up-regulation occurred in CYP19A1 (p=0.070).

20 out of 40 investigated genes showed significant regulations in ovary (table 4). Regulations occurred for the following receptors: AR (p=0.028), ERβ (p=0.010), FSHR (p=0.028), IGF-1 R (p=0.003) and PR (p=0.028), LHR (p=0.004) and Flt-1 (p=0.050). In the angiogenic group, up-regulations were observed for FGF-2 (p=0.038), ANGPT-2 (p=0.015), and MMP-2 (p=0.007). The proliferative factors IGF-1 (p=0.026) and BMP-2 (p=0.005) and also the anti-

245 cancerogenic protein p53 were up-regulated (p=0.021). Significant down-regulations were
246 demonstrated for VEGF120 (p=0.021), VEGF164 (p=0.015) and TIMP-2 (p=0.028), for the
247 enzymes S5A1 (p=0.021) and HSD17B3 (p=0.024) and for INHA (p=0.003). Trends for up-
248 regulation could be observed in Smad2 (p=0.083) and c-jun (p=0.052), Alk-6 (p=0.083)
249 tended to be down-regulated (table 4).
250 Principal components analysis (PCA) for uterine endometrium and ovary was produced as
251 shown in figure 1-3 by plotting the normalized Cq values from regulated target genes of all
252 samples by their first and second principal component. In the PCA, close clusters represent
253 experimental groups, with arrange together making it possible to separate these groups due
254 to the response to the treatment. Black triangles represent samples of the control group, grey
255 crosses represent the samples of treatment group. In all single tissues, a distinct control
256 group could be seen separating from the animals of the treatment group, showing that there
257 was a multitranscriptional response to the treatment. Additionally, a clear assignment could
258 be observed for the combination of all tissues (figure 4). The best separation between the
259 control and the treatment group was achieved for ovary and the composition of all three
260 target organs making these possibilities to the most promising regarding biomarker research.
261
262 *3.3 Results from pre-pubertal Holstein Friesian calves*
263 In ovary of pre-pubertal Holstein Friesian calves, 23 of 42 measured target genes showed
264 significant regulations (table 5). Significant regulations of five gens could already be detected
265 in the carrier control group. Herein, an up-regulation of LHR (p=0.032) and down-regulations
266 of VEGF120 (p=0.054) and VEGF164 (p=0.030), PR (p=0.028) and CYP19A1 (p=0.025)
267 could be observed. Most regulations occurred in the one time treated group. Up-regulations
268 could be revealed for the receptors LHR (p=0.014) and Alk-6 (p=0.013), the proliferative
269 factor c-kit (p=0.055), IGFBP-2 (p=0.012) and STAR (p=0.053). In the angiogenic group,
270 FGF-1 (p=0.008), MMP-23B (p=0.026) and the anti-angiogenic inhibitor TIMP-2 (p=0.008)
271 were significantly up-regulated. In the steroid metabolism group, S5A1 (p=0.030), HSD17B3
272 (p=0.034) and HSD17B8 (p=0.022) were up-regulated. HSD17B11 showed just a trend for

up-regulation in the one time treated group (p=0.064), but a statistical significant up-regulation in the three times treated group (p=0.031). Trends for up-regulation could be obtained for Flk-1 (p=0.069), ANGPT-2 (p=0.09), Rb-1 (p=0.095) and HSD3B1 (p=0.097). A trend for down-regulation could be seen for AR (p=0.071). In the three times treated experimental group HSD17B11 (p=0.031), MMP23B (p=0.043) and MMP-2 (p=0.016) were significantly up-regulated. A trend for up-regulation could be observed for ERα (p=0.098), PR (p=0.082) and BMP-2 (p=0.063).

In the PCA of ovarian tissue for pre-pubertal calves, all significantly regulated genes in all four experimental groups were plotted (figure 5) by their first and second principal component. Circles represent samples of the control group, crosses display samples of the carrier control group, triangles display the samples of 1x treated group, squares represent samples of the 3x treated group. Obviously, no separation could be seen between the two control and the two treatment groups making it debatable, if there was a response to the anabolic treatment on transcriptional level.

4. DISCUSSION

Several biochemical pathways could be shown to be differentially regulated on transcriptional level under the influence of an anabolic combination of an androgen and an estrogen in uterine endometrium and ovary of Nguni heifers. It is generally known that anabolic steroids and especially estrogens cause a trophic response in the reproductive tract [28]. Coincidently, several factors taking place in proliferative and anti-apoptotic events were regulated in the actual study. In ovary and uterus, AR is thought to play a role in physiological proliferation and also in uncontrolled cell growth during tumorgenesis [12]. There are few data concerning AR gene expression in bovine reproductive tract, but estrogens were shown to induce AR expression in rat uterus to mediate the uterotrophic effect [29;30]. Our data suggest that AR is targeted by anabolic steroids in bovine uterus and ovary triggering cell growth. Concordantly, TNFα, CEBPD and c-fos, which were shown to be implicated in cell turnover under the influence of steroid hormones [9;10;12;31;32], were up-regulated in Nguni heifers indicating a higher proliferation rate. While cell growth was induced, a parallel inhibition of apoptotic and tissue degrading factors occurred. BMP-2 and BMP-4 as well as CASP3 are known to cause apoptosis in many different target cells and have been proven to be implicated in tissue remodeling of the cycling uterus [33;34]. The protease CTSB plays a role in the degradation of extracellular matrix and the catabolism of intracellular proteins and is therefore physiologically involved in the tissue remodeling of the cyclic uterus [15]. The down-regulation of these factors propose an inhibition of tissue breakdown in the treated animals. In ovary, an up-regulation of the p53 tumour surpressor gene could be detected. p53 is known to be an inhibitor of caspase activity [14;35]. Possibly, caspase-dependent apoptosis in ovary is inhibited by the action of p53. Unlike that, the expression of IGF related factors illustrated another situation. It is generally known that IGF-1 is one of the major growth factors implicated in the proliferation of the uterus [36;37]. The present study showed a down-regulation of IGF-1R and an up-regulation of IGFBP-3 indicating a lower responsiveness and an inhibitory effect towards IGF-1. These regulations might demonstrate a protective adaption mechanism of the organism to prevent the

315 paracrine action of the plentiful IGF-1 originating from liver [42]. Contrarily, up-regulations of
316 IGF-1 and IGF-1R occurred in ovary rather pointing to strong proliferative effects. These
317 results also demonstrate the tissue specific response of the organism to the application
318 anabolic steroids.
319 A possible adaption mechanism of the organism to the anabolic treatment might also be
320 observed in the regulation of factors implicated in ovarian steroid synthesis in heifers.
321 HSD17B3 and S5A1, whose enzymatic reactions are directed towards the generation of
322 active steroid hormones, are down-regulated. INHA is known to be a suppressor of FSH,
323 whose secretion from the pituary is crucial for steroid synthesis, and its up-regulation could
324 therefore be part of the negative feedback mechanisms [20]. These data suggest an
325 inhibition of endogenous hormone synthesis and formation of active hormones as a negative
326 feedback response to exogenous application of anabolics. In pre-pubertal calves the up-
327 regulation of HSD17B8, which is responsible for the degradation of E2 into the lower active
328 estrone [5], could be estimated as an induction of decomposition of the applied exogenous
329 hormones. However, the enzymes HSD17B3 and S5A1 are induced not reflecting the
330 possible protective mechanism, which was hypothesized in Nguni heifers. This regulation
331 pattern gives no clear mark on a trend in steroid hormone synthesis in the pre-pubertal
332 ovary.
333 In ovary, VEGF and the VEGF-receptor Flt-1 are mainly implicated in the formation of new
334 capillary networks during the physiological process of angiogenesis [3]. The expression of
335 VEGF is mainly stimulated by LH secreted from the pituitary [3]. The observed down-
336 regulation of LHR in the ovary of heifers and the following lower responsiveness of this organ
337 towards LH may be responsible for the down-regulation of the VEGF isoforms and their
338 receptor Flt-1. The resulting inhibition of follicular maturation could partly be responsible for
339 reproductive perturbations like the delayed onset of puberty shown in pre-pubertal animals or
340 the predisposition for non-ovulatory estrus observed in mature cows under the influence of
341 steroidal growth promoters [38]. The changes in gene expression observed in the actual
342 study could also be accounted to mimic the state of regression of the corpus luteum (CL)

characterized by a down-regulation of VEGF and an up-regulation of FGF-2 and ANGPT-2 [39]. Thereby, these results could possibly indicate a degrading effect of anabolic steroids on the CL and follicle. In general, there might be an inhibitory effect on angiogenic processes under the influence of anabolic steroid hormones.

In pre-pubertal calves several pro-angiogenic factors like MMPs and FGF-1, as well as the mediating receptors Flt-1 and Alk-6 were up-regulated. This would point to an increase in angiogenic events after the application of exogenous hormones. However, also the anti-angiogenic inhibitor TIMP-2 and the blood vessel degrading factor ANGPT-2 are up-regulated prohibiting an explicit conclusion on angiogenic events. Considering the pour on study, most of the results in ovary are hardly to discuss. Even though the same genes were regulated compared to the ovary of Nguni heifers, the direction of the regulation differed, which was obvious especially in receptors (AR, PR, LHR), but also for steroidogenic enzymes (HSD17B3, S5A1). This could possibly be explained by the different hormonal status of calves and heifers. Accessorily, the reproductive tract of the calves might show no responsivity to the exogenous application of steroid hormones due to the rudimentary developmental stage. A similar hypothesis has been introduced by Caccicatore et al. [40], who observed no effect of hormone administration on different steroid target genes in pre-pubertal animals. Also, different anabolic preparations as well as different application modes (long lasting implants versus pour on) may cause different transcriptional responses in the treated animal. Especially, the route of application may be a reason for the unexpected results as rare valid data exist concerning the pharmacokinetics of steroid esters after pour on treatment. In the course of this study, co-workers investigated the occurrence of the applied steroid hormone esters in hair and plasma and were able to find the applied steroid esters in hair [41]. However, concentrations were under the detection limit after 5-7 weeks (1x treatment) and 9-11 weeks (3x treatment), respectively. Furthermore, no hydrolysates of the applied substances could be detected in plasma. These results indicate that the administered steroid esters compass hair via sweat or sebum excretion at the surface of the skin and reach the blood stream only in negligible amounts. Also, the short duration of the

371 drug effect could be proven. At the time point of slaughter, the applied substances have
372 already been eliminated from the organism, which could possibly be a reason for the
373 absence of specific gene expression changes [41].
374 Independently of the gene expression results, compared to the control group an anabolic
375 effect of the treatment was visible on the phenotype level by comparing weight gain and
376 carcass weight (unpublished data). Herein, the highest differences could be observed
377 between the control animals and animals from the 3x treatment group on days 28, 63 and 91
378 after beginning of the treatment. Also, the carcass weight at slaughter was significantly
379 higher in the 3x treated group. No differences could be detected between the two treatment
380 groups. These data demonstrate that an anabolic effect using pour on application becoming
381 manifest in significantly increased weight gain might not be visible at the gene expression
382 level and might therefore remain undetected, when using transcriptomics for surveillance of
383 anabolic misuse.
384 For biomarker research, results from biostatistical evaluation concerning pattern recognition
385 showed clearly that it would be necessary to establish different biomarkers for specific
386 treatment regimes and different age classes of animals, as the PCA of the ovary results
387 obtained from pre-pubertal calves indicated that the measured genes were not adequate as
388 a certain pattern to divide between treated and untreated animals. Contrarily, the PCA
389 conducted for all tissues from post-pubertal Nguni heifers demonstrated a distinct separation
390 between the control and the treatment group. These results give a first hint that it would be
391 possible to establish a gene expression pattern for the detection of anabolic misuse in adult
392 animals.
393

5. CONCLUSION

The observations gained in the actual study indicate a stimulation of cell turnover in the reproductive tract of post-pubertal Nguni heifers characterized by the induction of different transcription and proliferative factors and the inhibition of pro-apoptotic factors. In ovary, anti-angiogenic effects were estimated, which could in part be related to the well know disturbances in fertility following anabolic treatment.

The ambiguous transcriptional regulations in ovary of calves may be due to the non-responsiveness of the immature reproductive tract. The differences in gene expression compared to Nguni heifers could be explained by the different hormonal status of pre- and post-pubertal animals and the differences in the application routes. The application of PCA for pattern recognition demonstrated the possibility to establish a gene expression pattern, which could be used as biomarker to detect the illegal application of exogenous hormones in post-pubertal heifers for uterine endometrium and ovary. Pre-pubertal animals seem to be less suitable for surveillance of anabolic treatment using transcriptomics, as it was not possible to discover a convenient gene expression pattern using PCA in female Holstein Friesian calves.

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411

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415

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Figure 1: Slight separation between treated and non-treated heifers after PCA evaluation on the basis of 9 regulated target genes measured in uterine endometrium corpus;

▼= control group, × = treatment group

Figure 2: Slight separation between treated and non-treated heifers after PCA evaluation on the basis of 12 regulated target genes measured in uterine endometrium horn;

▼= control group, × = treatment group

Figure 3: Distinct separation between treated and non-treated heifers after PCA evaluation on the basis of 23 regulated target genes measured in ovary;

▼= control group, × = treatment group

Figure 4: Distinct separation between treated and non-treated heifers after PCA evaluation on the basis of 44 regulated target genes measured in reproductive tissues;

▼= control group, × = treatment group

Figure 5: No separation on the basis of 23 regulations measured in ovary of Holstein Friesian calves after PCA evaluation;

○= control group, + = carrier control group, ▼= 1x treated group, ■ = 3x treated group

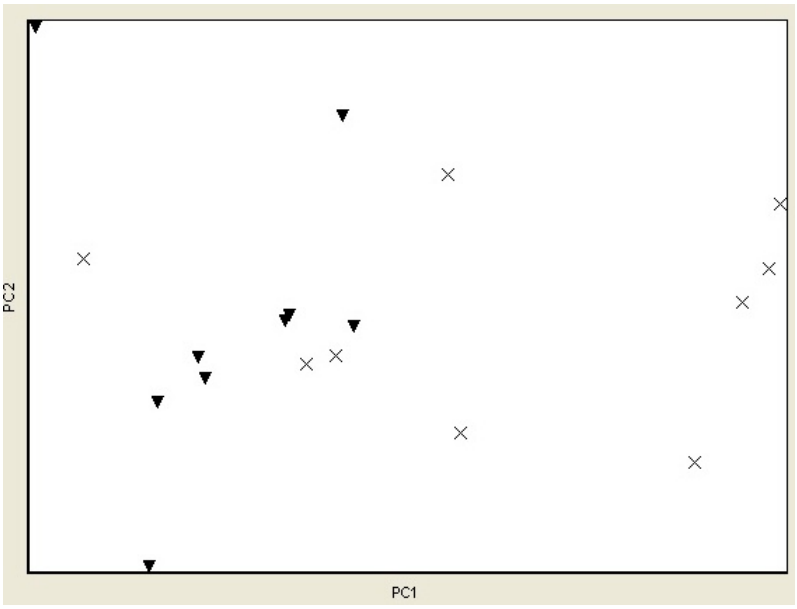

Figure 1: Slight separation between treated and non-treated heifers after PCA evaluation on the basis of 9 regulated target genes measured in uterine endometrium corpus;
▼= control group, × = treatment group

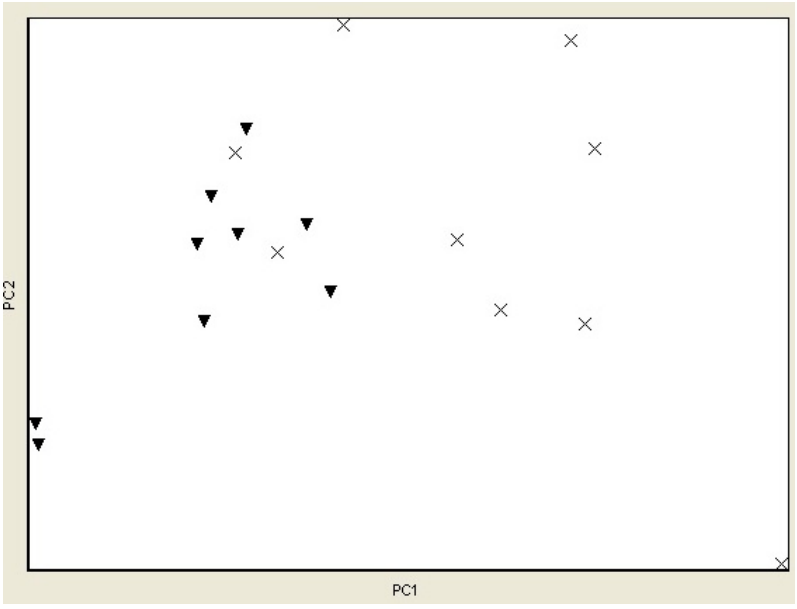

Figure 2: Slight separation between treated and non-treated heifers after PCA evaluation on the basis of 12 regulated target genes measured in uterine endometrium horn;
▼= control group, × = treatment group

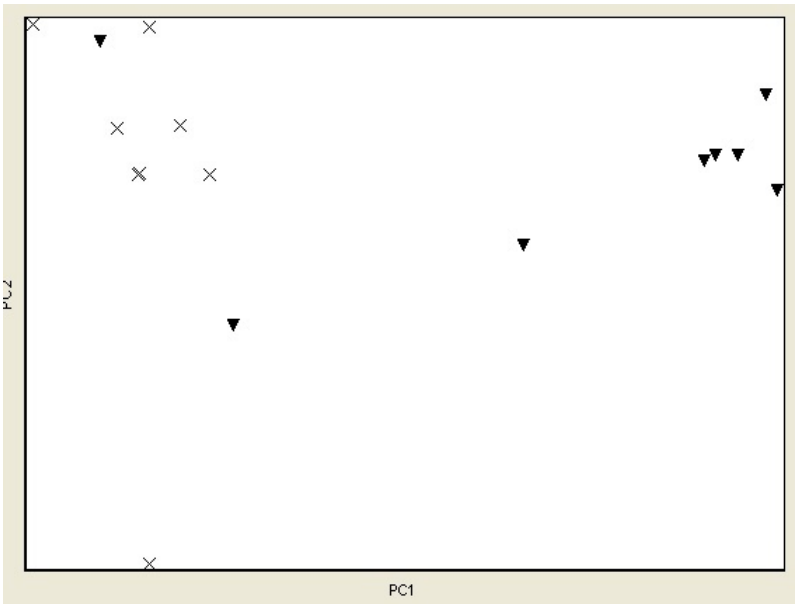

Figure 3: Distinct separation between treated and non-treated heifers after PCA evaluation on the basis of 23 regulated target genes measured in ovary;
▼= control group, × = treatment group

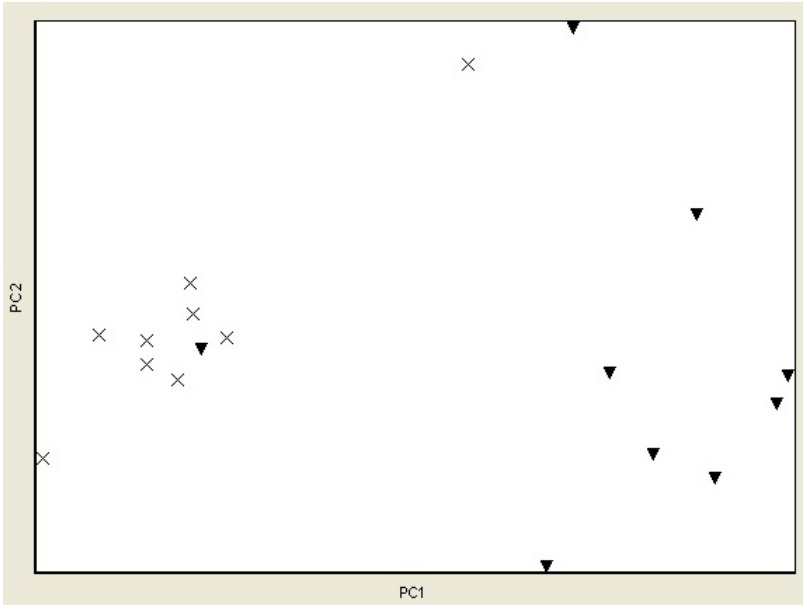

Figure 4: Distinct separation between treated and non-treated heifers after PCA evaluation on the basis of 44 regulated target genes measured in reproductive tissues;
▼= control group, × = treatment group

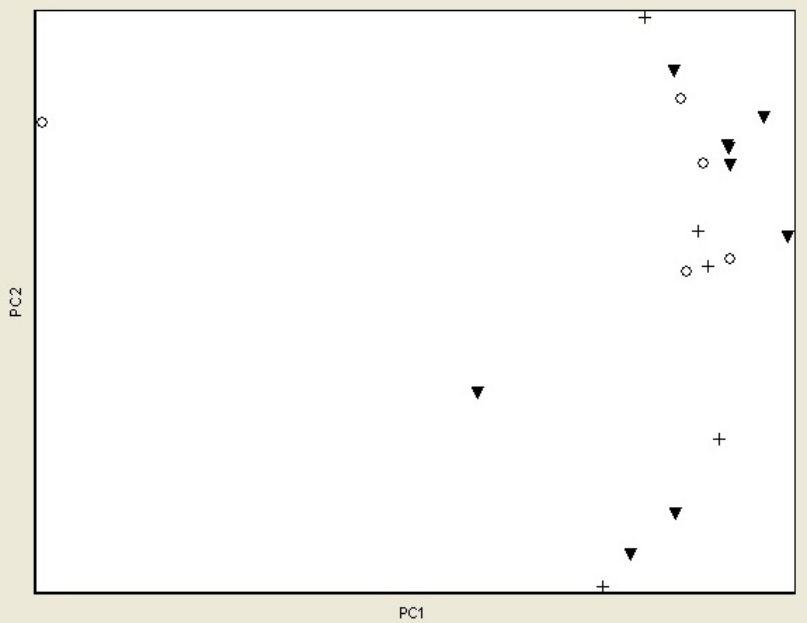

Figure 5: No separation on the basis of 23 regulations measured in ovary of Holstein Friesian calves after PCA evaluation;
○= control group, + = carrier control group, ▼= 1x treated group, ■ = 3x treated group

Table 1: Primer sequences of target genes measured in uterine endometrium (U) and ovary of Nguni heifers (O_H) and in ovary of Holstein Friesian calves (O_C) with the accordant annealing temperatures (T_M), product length (bp) and accession numbers; primers without accession numbers have been obtained from coworkers

Gene		sequence [5´→3´]	T_M	product lenght [bp]	Accession No.	
UBQ	for	AGA TCC AGG ATA AGG GAA GGC AT	60°C	198	Z18245	U, O_H,O_C
	rev	GCT CCA CCT CCA GGG TGA T				
H3	for	ACT GCT ACA AAA GCC GCT C	60°C	233	NM_001014389	U, O_H,O_C
	rev	ACT TGC CTC CTG CAA AGC AC				
AR	for	CCT GGT TTT CAA TGA GTA ACC GCA TG	60°C	172	AY862875	U, O_H,O_C
	rev	TTG ATT TTT CAG CCC ATC CAC TGG A				
ERα	for	AGG GAA GCT CCT ATT TGC TCC	60°C	234	AF177936	U, O_H,O_C
	rev	CGG TGG ATG TGG TCC TTC TCT				
ERβ	for	GAG ATA TTC TTT GTG TTG GAG TTT	60°C	164	NM_174051	U, O_H,O_C
	rev	CTT CGT GGA GCT CAG CCT GT				
PR	for	ACC AGC CCT ATC TCA ACT ACC	60°C	186	XM_583951.4	U, O_H,O_C
	rev	TAT GCT GTC CTT CCA TTG CCC				
LHR	for	CAG TGT GCT CCT GAA CCA GA	60°C	192	NM_174381	O_H,O_C
	rev	GTC TGC AAA GGA GAG GTT GC				
FSHR	for	AGTTGCCCTTTTTCCCATCTTTGG	64°C	150	NM_174061.1	O_H,O_C
	rev	TAGCAGCCACAGATGACCACAA				
GHR	for	CCA GTT TCC ATG GTT CTT AAT TAT	60°C	136	NM176608	U
	rev	TTC CTT TAA TCT TTG GAA CTG G				
GRα	for	TTC GAA AAA ACT GCC CAG C	62°C	194	AY238475	U, O_H
	rev	CAG TGT TGG GGT GAG TTG TG				
IGFR	for	CCC AAA ACC GAA GCT GAG AAG	60°C	314	X54980	U, O_H,O_C
	rev	CAT CCT CCT CGC ATC TCT TC				
EGFR	for	AAC TGT GAG GTG GTC CTT GG	60°C	169	AY486452	O_H,O_C
	rev	AAA GCA CAT TTC CTC GGA TGT CT				
ALK-5	for	CAG GGA AGA ACG TTC ATG GT	60°C	128	AF317296	O_H,O_C
	rev	CCA ACC AAA GCT GAG TCC AT				
ALK-6	for	GCC TGT TGT CAC CTC TGG AT	60°C	106	Z23143	O_H,O_C
	rev	CCT TTC TGT GCA GCA TTC AA				
BMPR II	for	CAA AGA TTG GCC CTT ATC CA	60°C	109	AJ534390	O_H,O_C
	rev	CTG GAC ATC GAA TGA TCT GA				
Flk-1	for	GCT TCT ACC AGG ACA CTG ACA T	60°C	144	X94298	U, O_H,O_C
	rev	AAC ACG GAA TCA CCA CCA CAG TT				
Flt-1	for	ATG ACC GAA GGG AAG AAG GTG	60°C	193	XM_001249768	U, O_H,O_C
	rev	TGA CTG TTG TCT CGC AGG TC				
MMP-2	for	CCC AGA CAG TGG ATG ATG C	60°C	237	NM_174745	U, O_H,O_C
	rev	TTG TCC TTC TCC CAG GGT C				
TIMP-2	for	GGG TCT CGC TGG ACA TTG	62°C	255	NM_174472	U, O_H,O_C
	rev	TTG ATG TTC TTC TCC GTG ACC				
MMP-23B	for	CGC GCT ACA GCT GGA AGA AAG GC	62°c	163	NM_001038556	O_C
	rev	ACA GCT CGT CCT GCG ATA GT				
FGF-1	for	TTG TAC GGC TCA CAG ACA CC	60°C	169	NM_174055	U, O_H,O_C
	rev	CTT TCT GGC CGA TGT GAG TC				
FGF 2	for	AGC CTT GCA ACT CTG CTT GT	60°C	210	NM_174056.3	U, O_H,O_C
	rev	CGA ATT CAG ATC CCT CCT GA				
FGF-7	for	GAC ATG GAT CCT GCC AAG TT	60°C	129	XM_869016	U, O_H,O_C
	rev	GGG CTG GAA CAG TTC ACA TT				

Table 1 *(continued*

Gene		sequence [5´→3´]	T_M	product lenght [bp]	Accession No.	
ANGPT-1	for	TCG GAG ATG GCT CAG ATA CAG	60℃	229	AF093573	U
	rev	CCA GCA GTT GTA TTT CAA GTC GA				
ANGPT-2	for	AAT TCA GTT CTC CAA AAG CAG C	60℃	216	NM_001098855	U
	rev	TCC ACC CGT TTC CAT GTC				
ANGPT-2	for	TTA TTC AGC GAC GTG AAG ACG G	62℃	187	NM_001098855	O_H,O_C
	rev	TAC AGC GAG TAA GCC TGA TT				
VEGFA	for	GGT GGA CAT CCT CCA GGA GTA	60℃	177	NM_174216.1	U
	rev	CTA TGT GCT GGC TTT GGT GAG				
VEGF 120	for	CCG TCC CAT TGA GAC CCT G	60℃	296	AB455252	O_H,O_C
	rev	CGG CTT GTC ACA ATT TTT CTT GTC				
VEGF 164	for	CCG TCC CAT TGA GAC CCT G	62℃	278		O_H,O_C
	rev	GCC CAC AGG GAT TTT CTT GC				
LDHA	for	GTG GCT TGG AAG ATA AGT GG	60℃	155	NM174099	U
	rev	ACT AGA GTC ACC ATG CTC C				
HK1	for	CAA GAC GCA CCC ACA GTA TCC	60℃	211	NM001012668	U
	rev	TCA CCT CCA GCA GCA TTT CCT T				
CAST	for	GAT CAG AAG TGC TGC TCC A	60℃	206	NM174003	U
	rev	GGA CTG TTT CCT CAT CTT ACC				
CTSB	for	GAT CTG CAT CCA CAG CCA	60℃	192	NM174031	U
	rev	ATG GAG TAC GGT CTG CAA CC				
CTSL	for	TCC ATA TCT TGC AAC GGA CAC TTT A	60℃	110	NM174032	U
	rev	CCT TCA TAA GGG CCT TCT CC				
IGF-1	for	CAT CCT CCT CGC ATC TCT TC	60℃	239	NM_001077828	U, O_H,O_C
	rev	CTC CAG CCT CCT CAG ATC AC				
IGF-2	for	ACC CTC CAG TTT GTC TGT GG	54℃	166	BC126514	O_H,O_C
	rev	ACA CAT CCC TCT CGG ACT TG				
IGFBP-2	for	AGC ATG GCC TGT ACA ACC TC	60℃	157	NM_174555	O_H,O_C
	rev	CCC TGC TGC TCG TTG TAG AA				
IGFBP-3	for	ACA GAC ACC CAG AAC TTC TCC T	60℃	202	NM174556	U, O_H,O_C
	rev	AGA AAC CCC GCT TCC TGC C				
BMP-2	for	CAG TAG GTG GGA GAG CTT CG	60℃	194	NM_0010099141	U, O_H,O_C
	rev	TGA CAA GCA AGG GCC TTA TCT GT				
BMP-4	for	GAG CTT CCA CCA CGA AGA AC	60℃	179	NM_001045877	U, O_H,O_C
	rev	TAC GAT GAA GC CCT GT CCC				
BMP-15	for	GCA GGC AGT ATT GCA TCT GAA G	60℃	250	NM_001031752	O_C
	rev	CAC TCT GAT CCA CCA GCT AC				
CYL D2	for	TGC AGA ACT TGC TGA CCA TCG	57℃	171	BC120199	O_C
	rev	GGT AAT TGA TGG CGA GAG GAA AG				
c-fos	for	CAG TGC CAA CTT CAT CCC AAC	60℃	189	NM_182786	U, O_H,O_C
	rev	CTG CCT CCT GTC ATG GTT TTC				
c-Jun	for	CGG CTA TAA CCC CAA GA	60℃	243	AF_069514	U, O_H,O_C
	rev	CCT GCT CAT CTG TCA CGT TC				
c-myc	for	TCT TGC GCC TAA ATT GAC CTA TTG	54℃	153	NM_001046074	U, O_H
	rev	GCC AAG GTT GTG AGG TTG TTC				
CEBPB	for	GCA CAG CGA CGA GTA CAA GA	60℃	152	NM_176788	U
	rev	GTT GCT CCA CCT TCT TCT GG				
CEBPD	for	ATC GAC TTC AGC GCC TAC ATC	62℃	101	BC133581	U
	rev	GCT TTG TGG TTG CTG TTG AAG AG				
Rb-1	for	CAA ATT CAG AGG CAC AAG CAA	62℃	179	NM_001076907	O_C
	rev	CTG GAA AAG GGT CCA GAT GAT				
RBBP-1	for	TTT CCA GGT CCA CTG GTC TC	60℃	226	NM_001034638	U
	rev	CTC AGA CAC CGA GCA AAT GAC				
c-kit	for	AAG TCC ATG CTG TCG AAG AA	60℃	185	XM_612028	O_C
	rev	TCT GCT GGC TGT TTT CCT TTA				

Table 1 *(continued)*:

Gene		sequence [5´→3´]	T_M	product lenght [bp]	Accession No.	
PHB	for	GTG AGC GAT GAC CTC ACA GA	60℃	163	NM_001034572.1	U
	rev	CAG CCT TTT CCA CCA CAA ATC T				
THBS	for	ACA CGA CTG CAA CAA GAA CGC	62℃	199	NM_174196	U
	rev	GGT TGG GGC AAT TAT CCT TTG T				
bcl-2	for	ATG ACT TCT CTC GGC GCT AC	62℃	245	XM_586976	O_H,O_C
	rev	CCG GTT CAG GTA CTC GGT CA				
bcl-xl	for	GGC ATT CAG CGA CCT GC	60℃	203	AF245487	U, O_H,O_C
	rev	CC TCC AAG TTG CGA TCC				
FAS	for	TGT TGT CAG CCT TGT CCT CC	60℃	174	U34794	U
	rev	GTT CCA CTT CTA GCC CAT GTT C				
Fas L	for	CAT CTT TGG AGA AGC AAA TAG	60℃	205	AB035802	U
	rev	GGA ATA CAC AAA ATA CAG CCC				
p53	for	ATT TAC GCG CGG AGT ATT TG GAC	60℃	174	NM_174201.2	U, O_H,O_C
	rev	CCAGTGTGATGATGGTGAGGA				
CASP3	for	GAC AGT GGT GCT GAG GAT GA	60℃	164	NM_001077840	U, O_H
	rev	CTG TGA GCG TGC TTT TTC AG				
CASP8	for	TAG CAT AGC ACG GAA GCA GG	62℃	294	DQ319070	U
	rev	GCC AGT GAA GTA AGA GGT CAG				
CYP11A1	for	CGG AAA GTT TGT AGG GGA CAT C	62℃	177	NM_176644	O_C
	rev	ACG TTG AGC AGA GGG ACA CT				
CYP19P1	for	TCA ACA GC GAG AG CTG GAA G	62℃	181	NM_174305	U, O_H,O_C
	rev	GGG GAT GCT TTG CAA TAA GAA ACA				
HSD3B1	for	TCC ACA CCA GCA CCA TAG AA	57℃	178	NM_174343	O_C
	rev	AAG GTG CCA CCA TTT TTC AGa g				
HSD17B1	for	CTC ATT ACC GGC TGT TCC TC	57℃	200	NM_001102365	O_C
	rev	ATG GAA TCT GCA TCC CTC ACg				
HSD17B3	for	CCC AAG CCA TTT CCT TAA CAC G	60℃	198	BC109700	O_H,O_C
	rev	ACA AAA GCC TTG GAA GCT GAA TAC				
HSD17B8	for	GGG CAT CAC CAG AGA TGA AT	60℃	228	NM_001046324	O_H,O_C
	rev	CAA TCA CTC CAG CCT TGG AT				
HSD17B11	for	GGT GAA GGC AGA AGT TGG AG	62℃	228	NM_001046286	O_H,O_C
	rev	AAG AAG GGG ACC CCA GTA TG				
S5A1	for	CCT TCC TAT TGG CGT TCA TCT TC	62℃	180	NM_001099137	O_H,O_C
	rev	ATT CAA ACA AGC CCC CTC TTG GT				
COX 2	for	GCC AGG GGA GCT ACG ACT A	60℃	247	NM_174445.2	U
	rev	AAG GAC AAT GGG CAT GAA ACT GTG				
C3	for	ATT GCC AGG TTC TTG TAC GGG	60℃	258	NM_001040469	U
	rev	GTC ACT GCC TGA TTG CAA GAT G				
C7	for	GGC GGT CAA TTG CTG TTT ATG G	60℃	232	NM_001045966	U
	rev	GGT CTG CTT TCT GCA TCC TC				
TGFβ	for	ACG TCA CTG GAG TTG TGC GG	60℃	267	XM5929497	U, O_H,O_C
	rev	TTC ATG CCG TGA ATG GTG GCG				
TNFα	for	CCA CGT TGT AGC CGA CAT C	60℃	197	NM173966	U, O_H,O_C
	rev	CCC TGA AGA GGA CCT GTG AG				
GDF-9	for	CAT CGG TAT GGC TCT CCA GT	60℃	122	NM_174681	O_H,O_C
	rev	ATG GCC AAA ACA CTC AAA GGA CT				
STAR	for	TGG AAA AGA CAC GGT CAT CA	57℃	154	NM_174189	O_C
	rev	CTG GGG CAT CTC CTC ATA GA				
Smad 2	for	ATG GTC GTC TTC AGG TGT CC	60℃	237	NM_001046218	O_H,O_C
	rev	GCA GTT CCG TTA GGA TCT CG				
LTF	for	ACC ATC TCC CAA CCT GAG TG	60℃	285	NM_180998	U
	rev	AAA GTT GCT GCC CTT CTT CAC G				
INH A	for	TAG TGC ACC CTC CAA GTT TC	60℃	239	NM_174094	O_H,O_C
	rev	GGT TGG GCA TCT CAT AC				

Table 2: Regulated genes in uterine endometrium corpus of Nguni heifers after treatment with TBA plus E2

functional group	gene	x-fold regulation		p-value	significance
		↑	↓		
receptors	AR	1,71		0,012	**
angiogenesis	FGF-7	1,62		0,088	
	VEGFA	1,62		0,044	*
proliferation/ apoptosis	IGFBP-3	2,69		0,085	
	CASP3		0,82	0,005	**
	c-fos	2,73		0,028	*
	BMP-4		0,61	0,020	*
	CEBPD	2,28		0,024	*
others	Complement C7	4,42		0,022	*

* = p<0.05; **= p<0.01

Table 3: Regulated genes in uterine endometrium horn of Nguni heifers after treatment with

functional group	gene	x-fold regulation		p-value	significance
		↑	↓		
receptors	AR	1,65		0,014	**
	IGF-1R		0,73	0,083	
angiogenesis	ANGPT-1		0,47	0,027	*
	MMP-2		0,65	0,057	
proliferation/ apoptosis	TNFα	5,05		0,018	*
	CASP3		0,72	0,00001	***
	FASL	1,99		0,049	*
	p53		0,72	0,076	
	BMP-4		0,64	0,041	*
protein metabolism	CTSB		0,63	0,033	*
others	Complement C7	6,92		0,001	***
	CYP19P1	1,87		0,070	

TBA plus E2

* = p<0.05; **= p<0.01, ***= p<0.001

Table 4: Regulated genes in ovary of Nguni heifers after treatment with TBA plus E2

functional group	gene	x-fold regulation		p-value	significance
		↑	↓		
receptors	AR	1.67		0.028	*
	ERβ	8.19		0.010	**
	LHR		0.29	0.004	**
	FSHR	3.61		0.028	*
	ALK-6		0.56	0.083	
	FLT-1		0.64	0.050	*
	IGF-1 R	2.03		0.003	**
	PR	2.20		0.028	*
angiogenesis	VEGF 120		0.34	0.021	*
	VEGF 164		0.21	0.015	*
	FGF-2	2.06		0.038	*
	ANGPT-2	2.32		0.015	*
	MMP-2	2.46		0.007	**
	TIMP-2		0.24	0.028	*
proliferation/ apoptosis	Smad 2	1.27		0.083	
	c-jun	2.00		0.052	
	p53	1.68		0.021	*
	IGF-1	2.34		0.026	*
	BMP-2	2.64		0.005	**
steroid metabolism	S5A1		0.53	0.021	*
	HSD17B3		0.61	0.024	*
others	INHA	4.67		0.003	**

= $p<0.05$; **= $p<0.01$

Table 5: Regulated genes in ovary of Holstein Friesian calves after treatment with hormone mix

fuctional group	gene	Carrier Control		1x		3x	
		x-fold	p-value	x-fold	p-value	x-fold	p-value
receptors	AR		n.s.	0.71	0.073		n.s.
	ERα		n.s.		n.s.	1.89	0.098
	PR	0.37	0.028		n.s.	1.93	0.082
	FLK-1		n.s.	3.75	0.069		n.s.
	LHR	1.78	0.032	2.06	0.014		n.s.
	ALK-6		n.s.	3.44	0.013		n.s.
angiogenesis	VEGF 120	0.78	0.054		n.s.		n.s.
	VEGF 164	0.70	0.030		n.s.		n.s.
	MMP-2		n.s.		n.s.	3.90	0.016
	MMP-23B		n.s.	1.63	0.026	1.67	0.043
	ANGPT-2		n.s.	1.90	0.090		n.s.
	TIMP-2		n.s.	3.78	0.008		n.s.
	FGF-1		n.s.	2.25	0.008		n.s.
proliferation	BMP-2		n.s.		n.s.	2.87	0.063
	IGFBP-2		n.s.	2.73	0.012		n.s.
	BMP-15		n.s.		n.s.	4.81	0.058
	c-kit		n.s.	1.93	0.055		n.s.
	RB-1		n.s.	1.72	0.095		n.s.
steroid synthesis	CYP19A1	0.26	0.025		n.s.		n.s.
	S5A1		n.s.	1.96	0.030		n.s.
	HSD3B1		n.s.	4.67	0.097		n.s.
	HSD17B3		n.s.	1.47	0.034		n.s.
	HSD17B8		n.s.	2.01	0.022		n.s.
	HSD17B11		n.s.	2.06	0.064	1.91	0.031
	STAR		n.s.	2.49	0.053		n.s.

Appendix IV

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ORIGINAL PAPER

Detectability of testosterone esters and estradiol benzoate in bovine hair and plasma following pour-on treatment

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Abstract The abuse of synthetic esters of natural steroids such as testosterone and estradiol in cattle fattening and sports is hard to detect via routine urine testing. The esters are rapidly hydrolysed in vivo into substances which are also endogenously present in urine. An interesting alternative can be provided by the analysis of the administered synthetic steroids themselves, i.e., the analysis of intact steroid esters in hair by liquid chromatography tandem mass spectrometry (LC/MS/MS). However, retrospective estimation of the application date following a non-compliant finding is hindered by the complexity of the kinetics of the incorporation of steroid esters in hair. In this study, the incorporation of intact steroid esters in hair following pour-on treatment has been studied and critically compared with results from intramuscular treatment. To this end animals were pour-on treated with a hormone cocktail containing testosterone cypionate, testosterone decanoate and estradiol benzoate in different carriers. The animals were either treated using injection and pour-on application once or three times having 1 week between treatments using injection and pour-on application. Animals were slaughtered from 10–12 weeks after the last treatment. Both hair and blood plasma samples were collected and analysed by LC/MS/MS. From the results, it is concluded that after single treatment the levels of steroid esters in hair drop to CCβ levels (5–20 μg/kg) after 5–7 weeks. When treatment is repeated two times, the CCβ levels are reached after 9–11 weeks. Furthermore, in plasma, no steroid esters were detected; not even at the low microgramme per litre level but—in contrast with the pour-on application—after i.m. injection, significant increase of 17β-testosterone and 17β-estradiol were observed. These observations suggest that transport of steroid esters after pour-on application is not only performed by blood but also by alternative fluids in the animal so probably the steroid esters are already hydrolysed and epimerized before entering the blood.

Keywords Liquid chromatography · Mass spectrometry · Hair · Testosterone ester · Estradiol benzoate · Pour-on treatment

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Introduction

Anabolic steroids are banned substances in the European Union but might still be illegally applied as growth promoters in cattle fattening [1, 2]. In the search for suitable sample matrices, bovine hair has shown to be an attractive sample matrix for prolonged detectability of residues of anabolic steroids and beta-agonists. Such substances can be incorporated into hair from blood via the hair follicle, incorporated from sweat via the hair shaft, absorbed from sweat on the outside of the hair shaft, and/or absorbed from

exogenic sources such as (environmental) contamination or intentional illegal pour-on treatment [3–6]. The potential of hair analysis for the determination of steroids and beta-agonists has been demonstrated in several papers [5, 7–18]. Natural steroids are usually administered as synthetic steroid esters but these are rapidly hydrolysed in vivo into natural steroids: following oral intake of testosterone undecanoate, the unchanged ester could be found in plasma from athletes for 6 h only [19]. In urine, it is hard to differentiate between the (metabolites of) endogenous natural steroids always present and the identical (metabolites of) natural steroids from the hydrolysed esters. In doping control, this problem is usually addressed by establishing the 17β-testosterone/17α-testosterone urinary ratio (so-called *T*/*E* ratio) [20] and/or the application of $^{13}C/^{12}C$ isotope ratio mass spectrometry [21]. In food surveillance, relatively high or low findings of 17β-testosterone and 17α-testosterone in urine are often ignored because of the lack of statistically valid reference data of naturally occurring background levels. An interesting alternative for inconclusive urine analyses in veterinary control can be provided by the analysis of the administered synthetic steroids themselves, i.e. the analysis of intact esters of natural steroids in hair. Gleixner and Meyer digested hair samples using dithiothreitol to break up the longitudinal chains of the keratin protein of the hair by reducing the disulfide bonds and applied that method to the determination of the natural steroids estradiol and testosterone [10]. Hooijerink et al. developed an LC/MS/MS method for the determination of intact estradiol benzoate in hair using the reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP) [22]. Meanwhile, that method has been further developed towards a true multiresidue screening method for all kinds of intact esters of testosterone, boldenone and estradiol [23] and adopted by other countries as well [24]. Thanks to this method, several cases of illegal use of synthetic esters of natural steroids were discovered in recent years.

Following a non-compliant finding of a synthetic ester of a natural steroid in hair, questions arise regarding a retrospective estimation of the date of illegal application might arise. In general, there will be no simple answers to that. When no preparations have been found, the route of administration remains unknown and might comprise injection, oral, sublingual, pour-on or other means. Moreover, several papers studying hair segments reported the lack of correlation between incorporation time and growth rate of hair [25–27]. This discrepancy is believed to be due to the fact that steroid esters are mainly incorporated via sweat and sebum excretion at the surface of the skin followed by diffusion into the hair fibre [25]. Apart from that, interindividual differences in metabolisation rates do occur of course.

Data about detectability in hair following pour-on application are rare. Anielski et al. [28] investigated a NorAndrosteDerm application containing prohormones of nortestosterone but intact esters were not studied. Hooijerink et al. [22] reported estradiol benzoate concentrations up to 8500 ng g^{-1} in bovine hair following pour-on application. Thieme et al. [6] compared transdermal and oral application of anabolic steroids and concluded that effective and durable concentrations of active steroids in blood could be obtained following the former treatment.

This study was designed to test the hypothesis whether the incorporation of intact steroid esters into bovine hair following pour-on treatment is detectable. The findings were critically compared with results from intramuscular treatment. To this end, animals were pour-on treated with a typical hormone cocktail containing testosterone cypionate, testosterone decanoate and estradiol benzoate in different carriers, or by intramuscular injection. Hair and blood samples were collected during several weeks and analysed for intact steroid esters by state of the art LC/MS/MS. Hair samples were taken by using a razor. The possibility of carry-over by using one razor to shave different animals was investigated as well.

Experimental

Chemicals, reagents and solutions

LC-C/MS-grade acetonitrile, water and methanol were obtained from Biosolve (Valkenswaard, the Netherlands). Formic acid, acetone, acetonitrile, ethanol, *n*-pentane, isooctane, dithiothreitol and Tris(hydroxymethyl)-aminomethane were obtained from Merck (Darmstadt, Germany). The reduction agent tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was obtained from Sigma (St. Louis, MO, USA). Estradiol benzoate (EB), testosterone cypionate (TC) and testosterone decanoate (TD) were obtained from Sigma-Aldrich Chemie b.v (Zwijndrecht, the Netherlands). *N*-methyl-*N*-trimethylsilyl-trifluoro(*o*)acetamide (MSTFA) was obtained from Alltech (Anaconda, Montana, USA), ammonium iodide was obtained from Fluka (Zwijndrecht, the Netherlands). The steroids 17α-testosterone and 17β-testosterone were obtained from Steraloids (Newport, Rhode Island, US). 17α-Estradiol was obtained from Diosynth (Morrisville, North Carolina, US), 17β-estradiol was obtained from Organon (Oss, the Netherlands) and the internal standards 17β-testosterone-d2 and 17β-estradiol-d3 were obtained from RIVM (Bilthoven, the Netherlands). The deuterium-labelled internal standards d3-estradiol benzoate, d3-testosterone cypionate and d3-testosterone decanoate were in-house-synthesised as described in [23]. The carrier solvents for pour-on application Ivomec® (mixture of isopropanol and Ivermectin B1a, B1b), DMSO (dimethyl sulfoxide Ph Eur), Mygliol® 840 (Triglycerida

saturate media Ph Eur), DEGMBE (diethyleneglycol monobutyl ether) and the carrier for injection Arachide oil were obtained from Spruyt Hillen b.v. (IJsselstein, the Netherlands). All other chemicals used were of analytical-reagent grade.

The derivatization reagent MSTFA++ for the free steroid analysis consisted of *N*-methyl-*N*-trimethylsilyl-trifluoro(*o*) acetamide/ammonium jodide/dithiothreitol (1,000/2/4; *v*/*w*/*w*). Individual stock solutions of the steroid esters were prepared by dissolving 10 mg of each compound in 10 ml of methanol. Individual stock solutions of the steroids were prepared by dissolving 10 mg of each compound in 10 ml of ethanol. Solid-phase extraction columns type Bond Elute LRC-C18 Phase 100 mg and 500 mg columns were obtained from Varian (Harbor City, CA, USA).

Equipment

The separation of the steroid esters was carried out using an ultra performance liquid chromatographic (UPLC) system, consisting of vacuum degasser, autosampler and a binary pump (Acquity UPLC system; Waters, Milford, MA) equipped with a reversed phase Waters acquity UPLC BEH C18 analytical column of 100×2.1 mm and 1.7 μm particle size. The gradient (solvent A, water–acetonitrile–methanol–formic acid (300:350:350:20, *v*/*v*/*v*/*v*); solvent B, acetonitrile–methanol–formic acid (500:500:20, *v*/*v*/*v*)) was: 0 min, 0% B; 0.5–6 min, linear increase to 100% B; 6–7.5 min 100% B. Injection volume was 40 μl. The UPLC system was connected to a triple-quadrupole mass spectrometer model Quattro Premier (Waters, Manchester, UK) equipped with an electrospray interface operating in the positive ion mode. The MRM transitions acquired for the different steroid esters of interest are summarised in Table 1.

The free steroids 17α-, 17β-estradiol and 17α-, 17β-testosterone were analysed by using GC-MS/MS. A Varian (Palo Alto, California, US) 1,200 L triple-quadrupole MS/MS spectrometer equipped with a CP8400 autosampler and CP-3800 GC was used. The GC column was a VF-17MS (*L*=30 m, id=0.25 mm, *df*=0.25 μm) obtained from Varian. The temperature programme used started at 110 °C (hold 1 min); increased at 20 °C/min to 240 °C (hold 1.5 min); increased with 1 °C /min to 243 °C followed by an increase of 25 °C/min to a final temperature of 340 °C (hold 2 min). The GC-MS/MS was operated in the EI ionisation mode. The MRM transitions acquired for the different steroids of interest are summarised in Table 2.

Samples

An animal trial has been conducted at the Physiology Weihenstephan, Technical University of Münich (Germany). The protocol has been approved by the ethical committee of the 'Regierung von Oberbayern'. Twenty veal calves (Holstein–Friesian) were obtained from identified sources at an age of 2 weeks. The animals were housed on straw and the different treatment groups were housed in separate pens. The animals were fed according to veal calf practise, using milk replacer and roughage, drinking water was ad lib.

Pour-on experiments were carried out using hormone cocktails containing three steroid esters. Prior to application the animals were shaven on the back from neck to tail. There, they were treated with 10 ml pour-on cocktail. In total, a set of four pour-on hormone cocktails were prepared and for each cocktail, a different carrier solvent was used. The hormone cocktails contained 25 mg of EB, 60 mg of TD and 60 mg of TC per 10 ml of carrier solvent. The carrier solvents used were Ivomec, DMSO, Mygliol 840 and DEGMBE.

Table 3 presents the application scheme. Five animals were not treated at all and were used as reference group. Four animals were treated at day 1, 7 and 14 (repeated treatment) with 10 ml of carrier solvent. Four animals were treated at day 1 with 10 ml of hormone cocktail; each animal was treated with a different carrier solvent. Four animals were treated on day 1, 7 and 14 (repeated treatment) with 10 ml of hormone cocktail. One animal was injected intramuscular in the neck on day 1 with a cocktail of 35 mg EB, 60 mg TD and 60 mg TC and one

Table 1 Steroid esters; specific ions monitored for LC-ESI(+)-MS/MS

Compound	MRM transition 1 (*m*/*z*)	Collision energy MRM transition 1 (eV)	MRM transition 2 (*m*/*z*)	Collision energy MRM transition 2	Rt in min
Estradiol benzoate	377>105	40	377>77	45	3.64
Estradiol benzoate-d3	380>105	20			3.63
Testosterone cypionate	413>97	25	413>107	25	5.46
Testosterone cypionate-d3	416>97	25			5.45
Testosterone decanoate	443>97	25	443>109	30	6.53
Testosterone decanoate-d3	446>97	25			6.53

Table 2 Free steroids; specific ions monitored for GC-EI-MS/MS

Compound	MRM transition 1 (m/z)	Collision energy MRM transition 1 (eV)	MRM transition 2 (m/z)	Collision energy MRM transition 2
17α-estradiol	416>326	−7.5	416>285	−10.0
17β-estradiol	416>326	−7.5	416>285	−10.0
17β-estradiol-d3	419>285	−10.0		
17α-testosterone	432>327	−15.0	432>209	−12.5
17β-testosterone	432>301	−15.0	432>209	−12.5
17β-testosterone-d2	434>211	−12.5		

animal was injected on day 1, 7 and 14 with the hormone cocktail. For the injection, arachide oil was used as the carrier. One animal was injected three times (day 1, 7 and 14) with arachide oil.

For pour-on application, the liquid-containing steroid esters (hormone cocktail) were spread out over the small (shaved) strip at the back of the animal from neck to tail. Hair samples were taken at least 20 cm off from the application zone. The first hair samples were taken before treatment. After 1 week (7 days) the second hair sample was taken at the next spot (see Fig. 1). The samples taken 35 days after treatment are taken at the same spot as the sample which was taken at day 1. Table 4 and Fig. 1 describe the 'hair sampling scheme' including the spots where the samples are taken, the number of days after first treatment and the weeks the hair had been growing at the specific spot. Each sample contained approximately 2–5 g of hair.

Hair samples were taken by using one razor but with one shaver head for each treatment group. To check for carry-over from one hair sample to the other due to the use of only one razor system, a contamination experiment was performed. The (worst case) contamination experiment was as follows: a piece of skin was treated (pour-on) using the steroid cocktail. The hair directly on the pour-on spot was collected by using a razor. The razor was cleaned dry by carefully removing the hair and blowing some air through the razor. No visible pieces of hair were left. The same razor was used to shave an untreated piece of skin. This hair sample was tested for containing residues of steroids esters.

Samples of blood were taken at days 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 42, 56, 70, 84 and 98. The samples were taken

Table 3 Application scheme

Animal group	Day of treatment	Animal id no.	Type of treatment
Reference group (n=5 animals)	–	175, 182, 185, 191, 193	No treatment
Reference group treated (3 times) with carrier solvents only	Day 1, 7 and 14	172	Injection with Arachide oil
		173	Pour-on with carrier solvent DEGMBE
		177	Pour-on with carrier solvent DMSO
		181	Pour-on with carrier solvent IVOMEC
		184	Pour-on with carrier solvent Miglyol
Treated once	Day 1	174	i.m. injection with hormone cocktail[a]
		178	Pour-on hormone cocktail in DEGMBE
		180	Pour-on hormone cocktail in DMSO
		183	Pour-on hormone cocktail in IVOMEC
		188	Pour-on hormone cocktail in Miglyol
Treated three times	Day 1, 7 and 14	171	i.m. injection with hormone cocktail
		179	Pour-on hormone cocktail in DEGMBE
		187	Pour-on hormone cocktail in DMSO
		190	Pour-on hormone cocktail in IVOMEC
		192	Pour-on hormone cocktail in Miglyol

[a] Hormone cocktail contents 25 mg of estradiol benzoate, 60 mg of testosterone decanoate and 60 mg of testosterone cypionate for each injection or for each 10 ml of carrier solvent

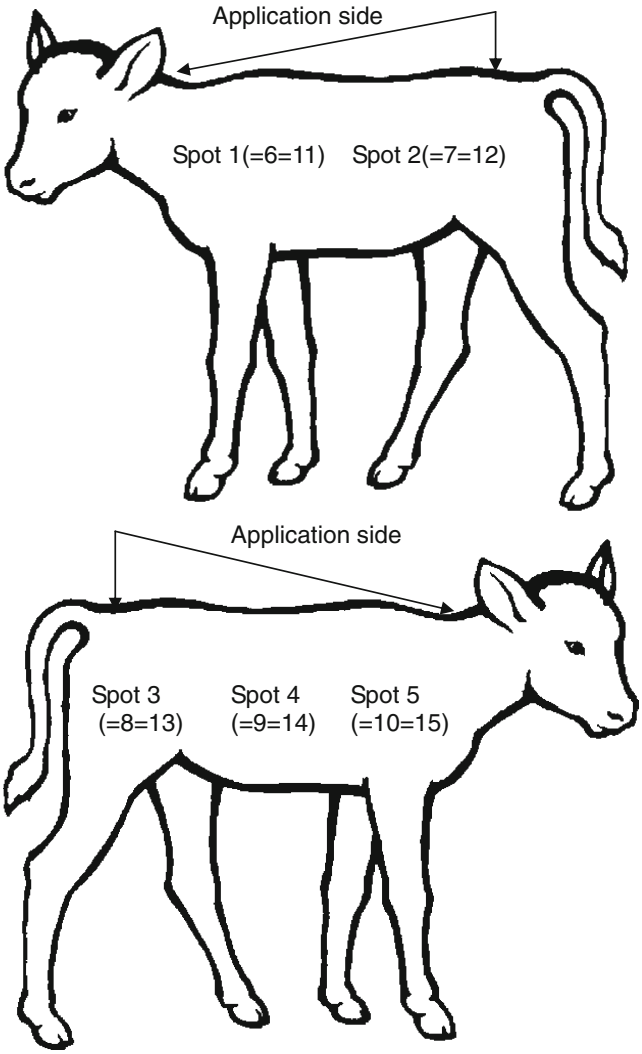

Fig. 1 Spots were the hair samples are taken (see also Table 4)

before each animal treatment. Blood samples were collected in EDTA Vacutainer, centrifuged and the plasma was used for the analysis. Hair and blood samples were stored at −20 °C.

Methods

Analysis of steroid esters in hair

Hair steroid esters The samples were treated as described in details by Nielen et al. [23] with some minor changes. The hair samples were analysed without using a washing step. Normally, the washing step with water is to wash away mud, etc. The samples were very clean so the washing step was omitted. In short, the procedure is as follows: the hair samples were cut into 0.5 cm pieces using a pair of scissors. Then, 500 mg was pulverised using a Mikro-dismembrator S ball mill. Two hundred milligrammes of the pulverised hair was weighed into a plastic tube and deuterium-labelled internal standards were added (10 ng/g hair). Digestion of the hair was performed by addition of 2 ml of 25 mM TCEP and incubation at room temperature for 1 h. The tube was shaken by using a 'head-over-head' shaker. After addition of 4 ml of methanol, the tube was centrifuged for 5 min at 1,700×*g*. Next, 4 ml of water was added and the mixture was applied to an activated C18 solid-phase extraction (SPE) column. The SPE column was conditioned by the addition of, respectively, acetonitrile, methanol and water. The column was washed with 2 ml of methanol/water (60/40, *v*/*v*) and eluted with 2 ml acetonitrile followed by 2 ml of ethyl acetate.

The SPE eluate was evaporated to dryness under a gentle steam of nitrogen gas at 40 °C and redissolved in 200 µl of LC solvent A, water–acetonitrile–methanol–formic acid (300:350:350:20, *v*/*v*/*v*/*v*). Finally, 40 µl was analysed by UPLC-MS/MS. For each steroid ester, two transitions (see Table 1) were monitored and for each deuterium-labelled analogue one transition was measured. The response factor was defined as the ratio between the sum of peak areas of the steroid ester transitions and the peak area of the transitions of the deuterium-labelled analogue.

For quantification, response factors versus concentration plots were constructed. To this end, seven blank hair samples were fortified with different concentrations of the steroid esters. The concentration of TC, TD ranged from 10 to 1,000 µg/kg and the concentration of EB from 2.5 to 250 µg/kg. The concentrations of the deuterium-labelled analogues were 50, 100 and 100 µg/kg for each sample of EB, TC and TD, respectively. The hair samples were analysed together with the calibration samples (Matrix

Table 4 Sampling scheme for hair samples (see also Fig. 1)

Spot	Days after first treatment	Weeks of grown hair
1	0 (before treatment)	8
2	7	9
3	14	10
4	21	11
5	28	12
6 (=1)	35	5
7 (=2)	42	5
8 (=3)	49	5
9 (=4)	56	5
10 (=5)	63	5
11 (=1=6)	70	5
12 (=2=7)	77	5
13 (=3=8)	84	5
14 (=4=9)	91	5
15 (=5=10)	98	5

Match Standards; MMS); concentrations were calculated using the linear regression method.

Analysis of steroid esters in plasma

The analysis of steroid esters in plasma was set up for this study and was based on the procedure described by Shackleton et al. [29]. To 1 ml of plasma, 5 ng of each internal standards and 4 ml of acetone/ethanol (1/1; *v*/*v*) were added. After intensive shaking and after centrifugation, the supernatant was transferred to a clean tube and the solvent was evaporated at 40 °C under a gentle stream of nitrogen. The residue was redissolved in 4 ml of methanol and 6 ml of water. The complete extract of 10 ml was applied to a preconditioned SPE column. The SPE procedure and the LC-MS/MS procedure were as described for the analysis of steroid esters in hair section. Because of the expected low concentrations of steroid esters in plasma the concentration range used for the calibration curve was 0–10 μg/l.

Analysis of the free steroids 17α-, 17β-estradiol and 17α-, 17β-testosterone in plasma

The samples were treated as described by van Tricht et al. [30]. In short, the procedure is as follows: to 1 ml of plasma, 0.5 ng of each internal standard and 1 ml methanol were added. The proteins were denaturated and after addition of 1 ml of water, the mixture was applied to a C18 SPE column. The SPE was preconditioned with 3 ml of methanol and 3 ml of water. After a washing step with water and acetonitrile/water (35/65; *v*/*v*) the analytes of interest were eluted with 3 ml of acetone. The eluate was collected and evaporated at 50 °C under a gentle stream of nitrogen and redissolved in 100 μl of methanol and 2 ml of Tris-buffer pH 9.5. Liquid–liquid extraction (LLE) was performed with 7 ml of *n*-pentane. This extraction step was repeated and the organic layers were collected and evaporated (see above). The residue was redissolved in 0.5 ml of ethanol and transferred into a derivatisation vial and the ethanol was evaporated. The dry residue was derivatised by adding 25 μl of MSFTFA++ followed by incubation of 1 hour at 60 °C. The derivatised mixture was evaporated and the residue was reconstituted in 25 μl of iso-octane; 4 μl was injected into the GC-MS/MS (pulse pressure 30 psi). The ion ratio was defined as the ratio between the area of the MS-MS transition 1 (see Table 2) of the steroid and the deuterium-labelled analogue. For 17α-estradiol and 17α-testosterone, the 17β- deuterium-labelled analogues were used. For quantification, ion ratios versus concentration plots were constructed. For quality control, five blank plasma samples were fortified with different concentrations of the steroid. The concentration of steroids ranged from 0 to 0.4 μg/l. The concentrations of the deuterium-labelled analogues were 0.2 μg/l for each sample. The plasma samples and quality control samples were analysed together with the calibration standards; concentrations were calculated using the linear regression method.

Results and discussion

There is not much information available regarding the pharmacokinetics of steroid esters administered to an animal by 'pour-on' application [26]. Therefore, it is of interest to know if the steroid esters finally reach the bloodstream. To collect information about the distribution of the steroid esters it was decided to analyse a selection of the plasma samples for containing steroid esters (EB, TC and TC). From literature, it is known [29] that after injection of steroid esters, the esters are hydrolysed and the free steroids are detected in the blood. To find out if the free steroid (17α-, 17β-estradiol and 17α-, 17β-testosterone) concentration in plasma also increases after 'pour-on' application, the free steroid concentration was measured in a sub selection of the plasma samples. The results of the analysis of hair samples and plasma samples are described below.

Determination of steroid esters in hair samples by LC-MS/MS

LC-MS/MS method performance

The analytical method for the determination of steroid esters in hair is described by Nielen [23]. The method was in-house-validated according to EU legislation 2002/657/EC [31] for 13 steroid esters—including EB, TC and TD. The validation was performed according to the guidelines for a confirmatory method. The CCα and CCβ were determined by the analysis of 20 different samples of hair with and without addition of steroid esters. The CCα of EB, TC and TD were respectively 2, 6 and 6 μg/kg. The CCβ of EB, TC and TD were respectively 5, 20 and 20 μg/kg. Recent publications [24, 32] demonstrate that the method is suitable for the analysis of steroid esters in bovine hair. To use the method as a quantitative method, some additional validation was performed. The linearity of the calibration curve was checked within the range of 2.5–250 μg/kg for EB and of 10–1,000 μg/kg for TC and TD. The coefficient of correlation (R^2) was better than 0.98 for all tested esters. The within-lab reproducibility was determined at the CCβ concentration level. In different sample series (analysed on different days), a (blank) sample fortified at CCβ level was

analysed. The %RSD was 20, 5 and 20% (n=9) for EB, TC and TD, respectively.

For this study—determination of the kinetics of the elimination—the obtained results from method validation are adequate. The CCβ-s are at or below 20 µg/kg, linearity is better than 0.98. Furthermore, deuterated internal standards are available for each steroid-ester to correct for recovery losses and the influence of the matrix resulting in within-lab reproducibility results of %RSD <25%.

Steroid esters in hair

Figures 2–6 present the concentration of the steroid esters measured in the samples of hair. The vertical lines at day 35 (after 5 weeks) divided the plot into samples of hair grown

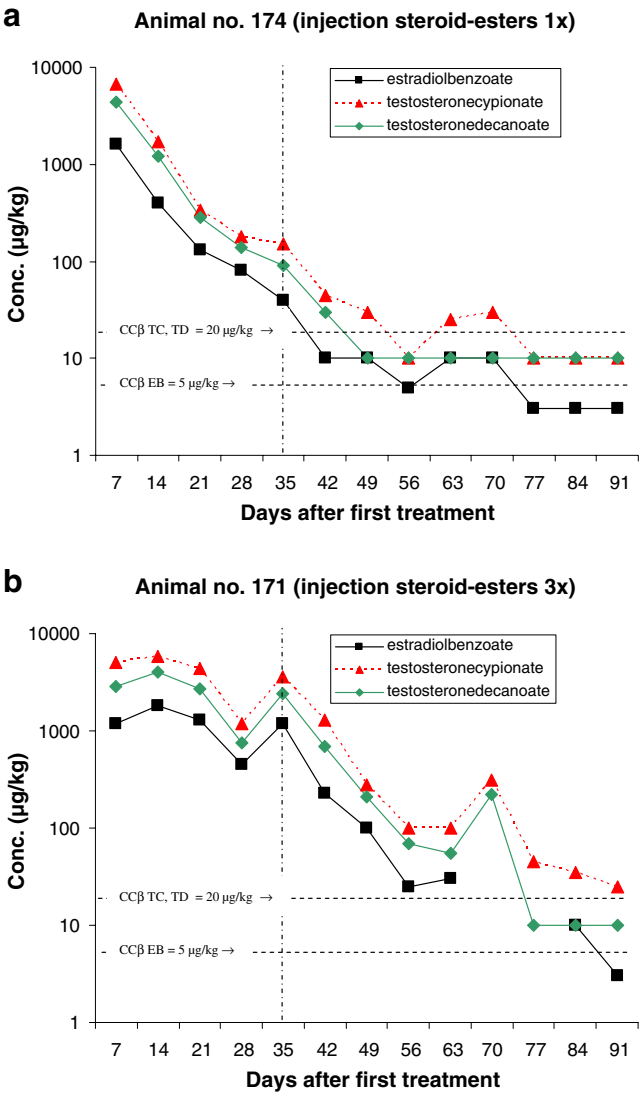

Fig. 2 Concentration of the steroid esters found in hair samples after intramuscular injection

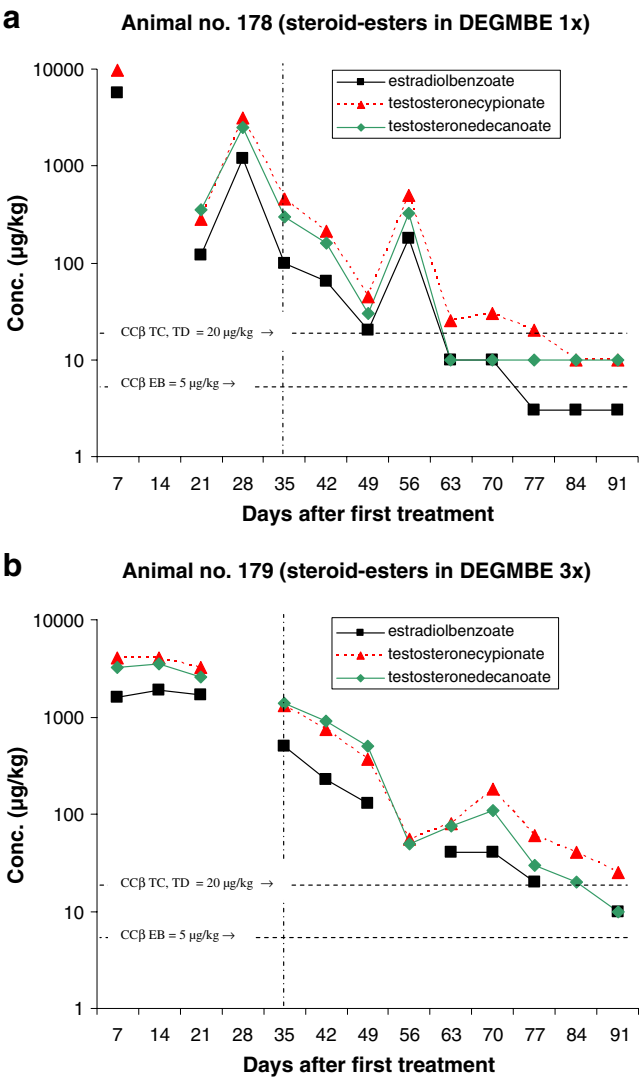

Fig. 3 Concentration of the steroid esters found in hair samples after pour-on treatment

for 8–12 weeks and samples grown for only 5 weeks (see also Fig. 1 and Table 4). The horizontal lines in the figures represent the CCβ-s of the steroid esters measured. Figure 2a represents the results of the animal injected once with the hormone cocktail and Fig. 2b represents the results of the repeated i.m. injection treatment. The treatment took place on days 1, 7 and 14. Note that the concentration scale is a logarithmic scale.

The results obtained after pour-on treatment are presented in the Figs. 3–6. The treatments presented in Figs. 3–6 differ only in the carrier solvent used DEGMBE, DMSO, IVOMEC or Miglyol. Figures 3a–6a present the single-treatment results and the Figs. 3b,–6b the repeated pour-on treatment results.

For monitoring the illegal use of steroid esters the CCβ concentration is most relevant because at and above that concentration, identification according to the EU criteria is

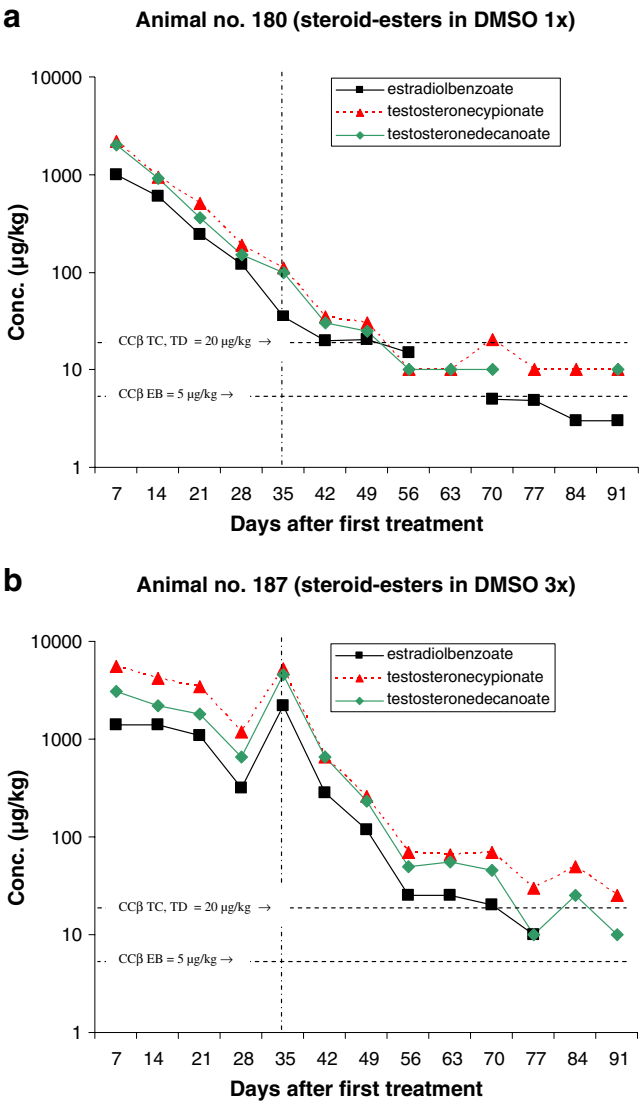

Fig. 4 Concentration of the steroid esters found in hair samples after pour-on treatment

possible in at least 95% of all non-compliant cases. For this study, it is important to establish how long after treatment the CCβ concentration level is reached; in other words, how long after treatment the hair sample is 'non-compliant'. All results are based on single-hair analysis. When no recovery of the internal standard was measured and/or when too many interfering peaks were measured, the quantification was not possible for the specific analyte/sample combination. These data are the missing points in the figures. The missing points at the higher concentration levels are mostly due to low recovery of the internal standards. The used method fits the purpose; however, due to the different sample pre-treatment steps necessary (pulverising, digestion, SPE), the absolute recovery is sometimes low. When the internal standard is not detected, the quantification is not possible. The missing points at or below the CCβ levels are, for most samples, due to observed interfering peaks most probably due to residues of some fatty compounds like sebum.

From the results presented in Figs. 2–6 some general conclusions can be drawn.

The maximum concentrations measured for all applications (pour-on and injection) are for TC with a maximum of 9,600 µg/kg by pour-on application in DMSO. In general, the TC concentrations are slightly higher than the TD concentration. Due to the concentrations applied EB shows the lowest concentrations in hair.

Table 5 presents how many days after treatment the CCβ concentration (lowest concentration resulting in 'non-compliant' results) levels are reached. In general, for single treatment, the CCβ levels are reached after 35–49 days with the only exception of the pour-on application in

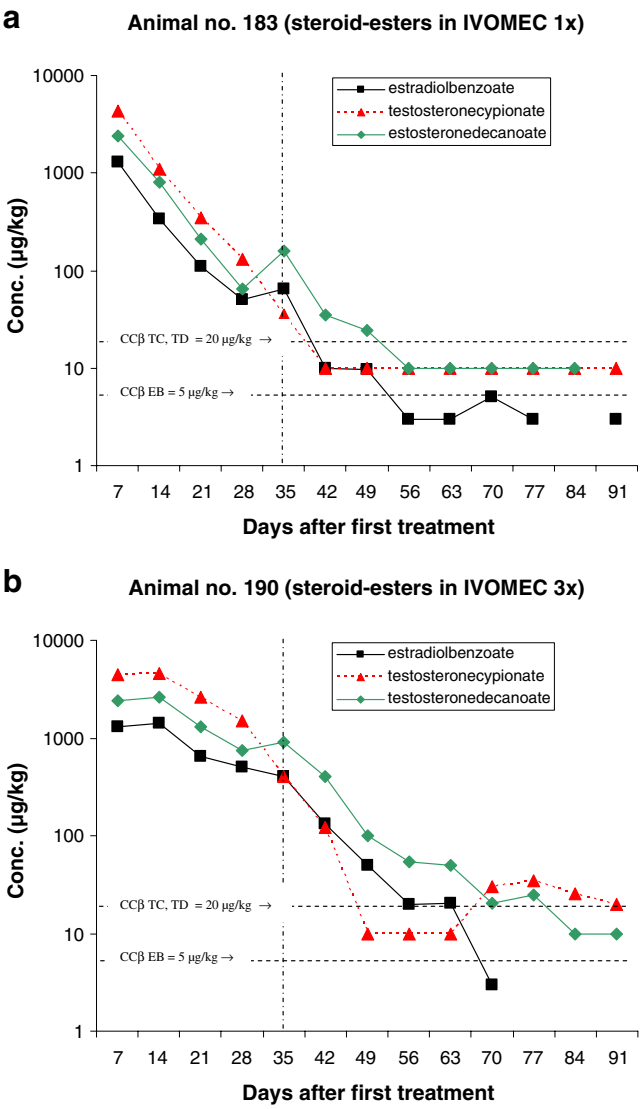

Fig. 5 Concentration of the steroid esters found in hair samples after pour-on treatment

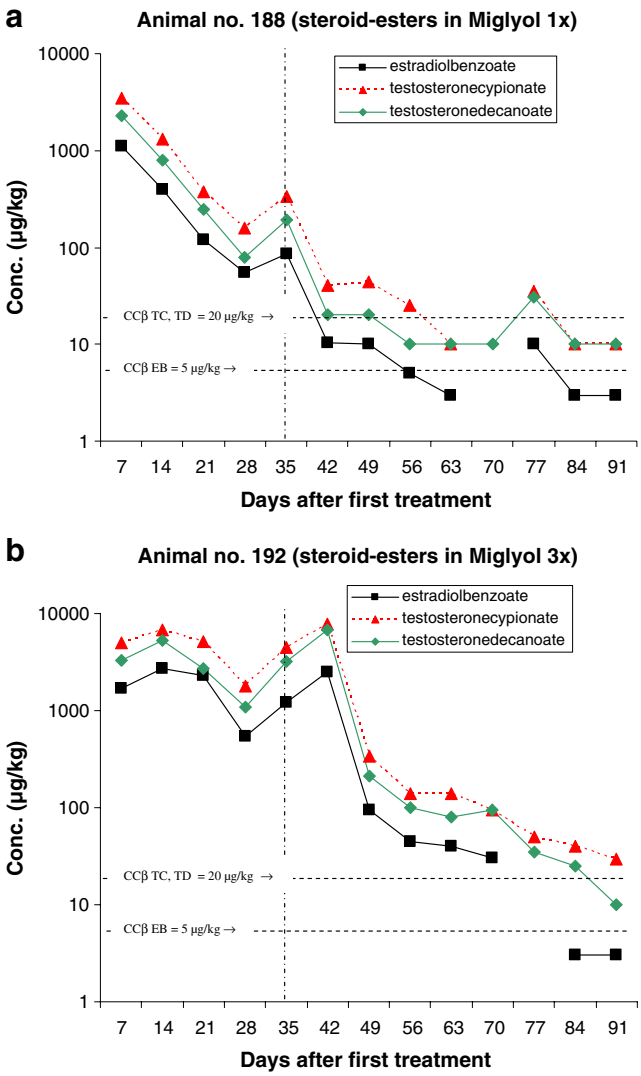

Fig. 6 Concentration of the steroid esters found in hair samples after pour-on treatment

DEGMBE for which the CCβ levels are reached after >56 days (8 weeks) after treatment. For the pour-on application in Miglyol, some relevant data points are missing but by extrapolating the elimination plot for TC it is expected that the CCβ is reached between 56 and 63 days after treatment.

For repeated treatment, the following conclusions can be drawn: it is obvious that the highest concentration levels of steroid esters are reached after the first treatment and that there is hardly any concentration differences between injection and pour-on application. The maximum elimination rate of steroid esters into the hair is probably reached already after the first treatment and so, additional treatments do not result in higher concentrations levels in the hair samples. The number of treatments do not have effect on the maximum concentration level in hair but on the number of days the concentrations are at a high level (>1,000 µg/kg). Consequently, after repeated treatment, it takes more time before the CCβ levels are reached. By repeated treatment, the high level is (in comparison with the single treatment) continued for 21–28 days then the concentrations started to decrease. For TC, the CCβ level is reached for all application after >91 days. The only exception is the pour-on application in IVOMEC for which TC reached the CCβ level after 43 days but these results are not very reliable because—rather unexpected—the TC concentration started to increase after 63 days. For the steroid esters EB and TD, the CCβ levels are reached at 63–77 days (9–11 weeks) after injection or pour-on treatment with the carrier solvent DMSO or IVOMEC. For the pour-on application in DEGMBE and Miglyol, slightly longer elimination times for EB and TD viz. 77–84 days (11–12 weeks) are observed.

It is difficult to draw conclusions about the comparison of injection and pour-on application. Only two animals were injected with the hormone cocktail. This experiment—in which one animal was injected once and one animal was injected three times—shows that the steroid ester concentrations in the hair samples collected after injection were roughly at the same level as after pour-on treatment. It seems that the application procedure does not affect the steroid ester elimination rate into the hair.

Note that the samples of hair collected before treatment (t=0 samples) did not contain any of the steroid esters at detectable concentrations. Based on the results obtained, it was decided not to analyse all the hair samples collected from the animals treated with only carrier solvent or untreated (reference group) animals. From these treatments, only the samples taken at week 0, 5, 10, and 14 were analysed. The samples did not contain any of the steroid esters at a detectable concentration.

Importantly, the sample of hair collected during the contamination (carry-over) experiment did not contain any of the steroid esters at a detectable concentration.

Determination of steroid esters in plasma by LC-MS/MS

A method was set up to monitor steroid esters in plasma. From literature, it is known that after application of 135 mg testosterone esters to a human body, the blood concentrations after 96 h is about 1 µg/l [29]. For that reason, the method was set up in the concentration range of 0–10 µg/l. The method was validated for qualitative analyses only but to each sample, the internal standards were added and the recovery of the internal standards (criteria S/N of internal standard has to be >3) was used as a sensitivity and recovery check. The lowest calibration point was at 1 µg/l and therefore the LOD was set for all steroid esters at this specific concentration level. All samples of plasma collected from the animals treated once and three times (injection and pour-on) with the hormone cocktail were analysed for containing

Table 5 Number of days after treatment the CCβ concentration levels are reached

	Injection	Pour-on application			
		DEGMBE	DMSO	IVOMEC	Miglyol
Single treatment					
Estradiol benzoate	35–42	56–63	42–49	35–42	35–42
Testosterone cypionate	49–56	70–77	49–56	35–42	>56
Testosterone decanoate	42–49	56–63	49–56	49–53	>49
Repeated treatment					
Estradiol benzoate	63–70	77–84	70–77	63–70	77–84
Testosterone cypionate	>91	>91	>91	42–49[a]	>91
Testosterone decanoate	70–77	84–91	70–77	77–84	84–91

[a] Unreliable results; concentration increases significantly after 63 days

steroid esters. In none of the samples detectable concentrations (> LOD) of steroid esters were monitored

As mentioned above, the method was partially validated and for that reason a selection of the plasma samples was send to the EU Community Reference Laboratory for hormones the RIVM (Bilthoven, the Netherlands) and were analysed under ISO/NEN 17025 accredited conditions for containing steroid esters. The sub selection consists of the following samples: plasma collected from Days 0 to 42 of the animal injected (3×), Days 0 to 14 of the animal injected 1× and the first five samples collected after pour-on application (1× and 3×) by using DEGMBE as the carrier solvent. Again, no steroid esters were detected. The LOD levels for EB, TC and TD for the LC-MS/MS method used were 0.2, 0.3 and 1 μg/l, respectively. That no steroid esters were detected in the plasma samples was not completely unexpected. Shackleton [29] already described that after application of 135 mg of testosterone esters to a human body the blood concentrations after 96 h is about 1 μg/l. In other words, rapid hydrolysis of steroid esters takes place as soon as the steroid esters reach the bloodstream.

Determination of free steroids in plasma by GC-MS/MS

GC-MS/MS method performance

The analytical method for the determination of free steroids in plasma and the validation of the method are described by van Tricht et al. [30]. The method was validated according to EU legislation 2002/657/EC [31] for 20 natural hormones—including 17α-, 17β-estradiol and 17α-, 17β-testosterone. For this study, the linearity of the calibration curve was checked for the 0–400 ng/l range. The coefficient of correlation (R^2) was better than 0.98. A summary of the method validation results is presented in Table 6. The measurement uncertainties based on within-laboratory-reproducibility (n=21) are for 17α- and 17β-estradiol 25 and 20%, respectively, and for 17α- and 17β-testosterone 40 and 46%, respectively.

For this study—determination of the kinetics of the elimination—the obtained results for method validation are adequate. The CCβ levels are at or below 16 ng/l for estradiol and 50 ng/l for testosterone, linearity is better than 0.98. The detection limits of this method are low enough to measure the concentration of natural hormones (endogenous hormones) in plasma. Furthermore, deuterated internal standards are available for each steroid to correct for recovery losses and the influence of the matrix. Although the observed uncertainties in the measurements of the free testosterone are relatively high, the method was useful for the determination of the elimination kinetic. The testosterone measurement showed high variability during this study, probably due to natural variation of testosterone concentrations.

Free steroids in plasma GC-MS/MS results

A selection of the plasma samples were analysed for 17α-, 17β-estradiol and 17α-, 17β-testosterone. The samples analysed were the samples collected from the animal injected with the hormone cocktail (1× and 3×). Injection is a traditional way of treating the animal with steroid esters. It is of interest to analyse the plasma samples collected after injection of the hormone cocktail and to compare the results with the results of the pour-on application. Since, only minor differences were observed in the steroid ester concentration of hair samples by using different carrier solvents for pour-on application, it was

Table 6 Validation paramaters for the GC-MS/MS analysis of steroids in plasma; adapted from [30]

Compound	CCα (in ng/l)	CCβ (in ng/l)
17α-estradiol	9	16
17β-estradiol	7	12
17α-testosterone	20	40
17β-testosterone	30	50

decided to analyse only one set of the plasma samples after pour-on application. For the analysis, the samples collected after pour-on application of the hormone cocktail with the carrier solvent DEGMBE (1× and 3×) were selected. These samples were selected because the corresponding hair samples contained the relatively high levels of steroid esters. Possibly, these high levels would correspond with high plasma levels resulting in detectable concentrations (above CCβ).

Figures 7 and 8 present the results obtained for the analysis of 17α-, 17β-estradiol and 17α-, 17β-testosterone in plasma samples. In Fig. 7, the results for 'injection' and in Fig. 8 the results for 'pour-on' are presented. Figures 7a and 8a are results obtained after single treatment; Figs. 7b and 8b are results obtained after repeated treatment. Note

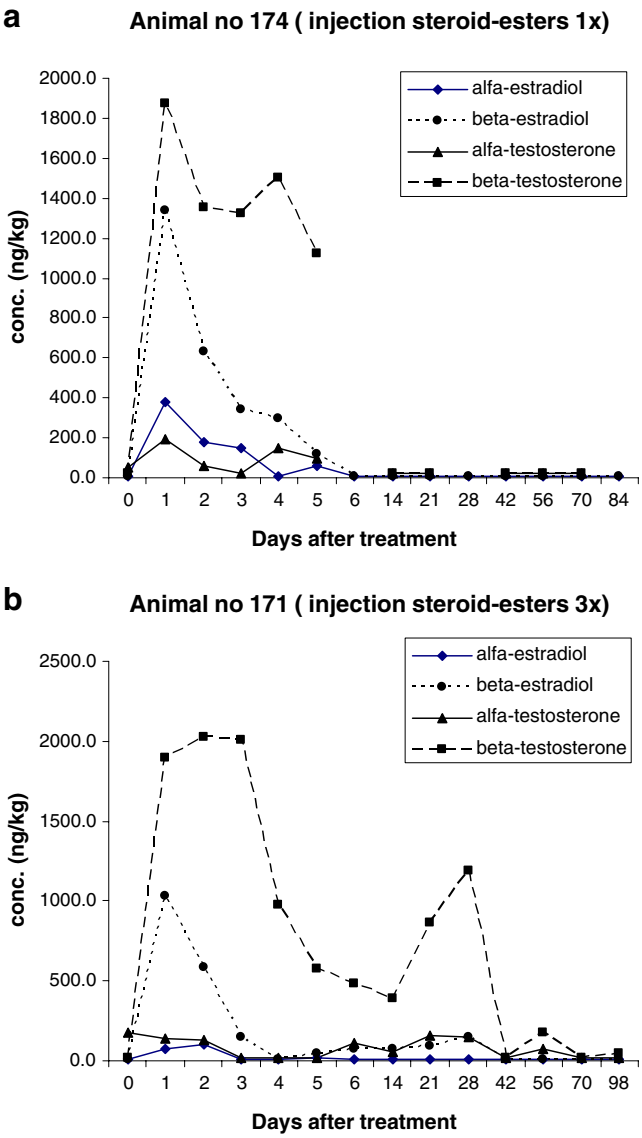

Fig. 7 Concentration of free steroids found in plasma after intramuscular injection

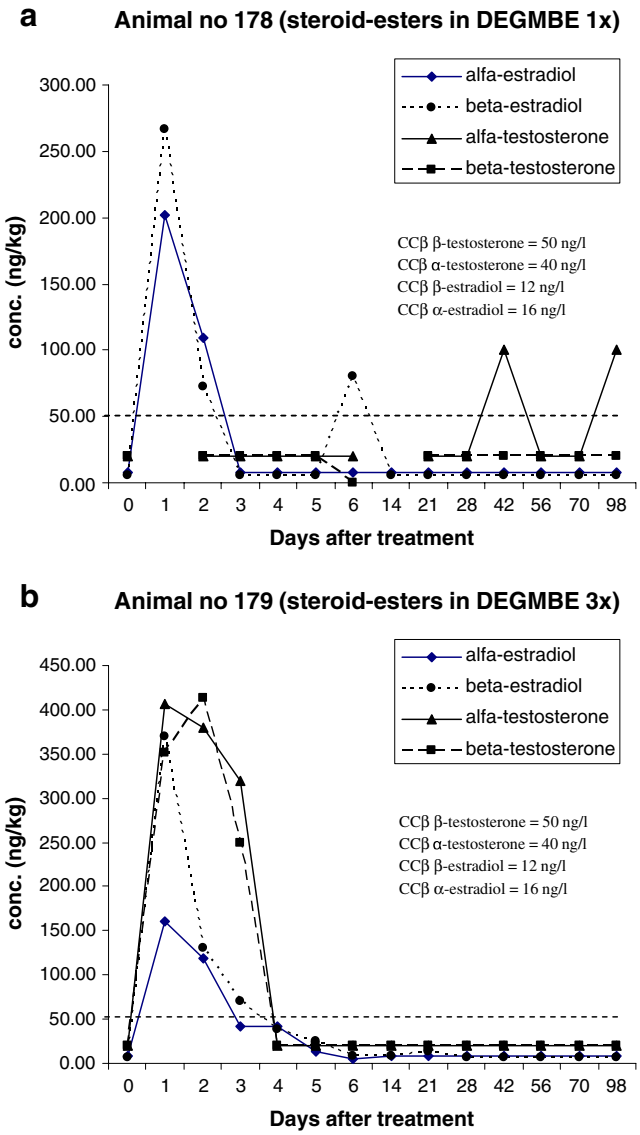

Fig. 8 Concentration of free steroids found in plasma samples after pour-on treatment

the non-linear time axis. The first plasma samples were collected just before the first treatment (Day 0) followed by 1, 2, 3, 4, 5 and 6 days after treatment. The next samples were collected at day 14, 21, 28 (2, 3 and 4 weeks after the first treatment) and at day 42, 56, 70, and 98 days (6, 8, 10 and 14 weeks after treatment). Samples taken at day 14 and day 21 were collected before the second and third treatments took place. When no recovery of the internal standard was measured and/or when too many interfering peaks were measured, the quantification was not possible for the specific analyte/sample combination. These data are the missing points in the figures. Especially around the CCβ levels, data points are missing due to some interfering peaks in these specific samples. Additional research is necessary to improve the robustness of the method.

Although it is difficult to draw conclusions based on one data set for injection and one data set for pour-on, some observations are worthwhile to mention. It is obvious that the concentration of the free steroids in plasma after injection is significantly higher than the concentrations of free steroids after pour-on application. It is of interest to see that after injection (Fig. 7b), the highest levels of free steroids are measured between 1 and 3 days after the first treatment and that the maximum concentrations reached for the 17β-forms are between 1,000 and 2,000 ng/l and for the 17α-forms are much lower, viz. 100 ng/kg. For the pour-on application (Fig. 8b), the highest levels of free steroids are also measured between 1 and 3 days after the first treatment, however, for this application, the maximum concentrations reached are much lower (maximum of 410 ng/l for 17β-testosterone). A second remarkable difference between injection and pour-on results is that for the pour-on application, there is no significant difference in maximum concentrations for the four steroids determined. In other words, the maxima reached for 17α-, 17β-estradiol and 17α-, 17β-testosterone are within the small range of 200–400 ng/l—in contrast, for injection, 17β-concentrations are approximately ten times higher than 17α-concentrations.

Regarding the steroid elimination time, it is clear that 5 days after repeated injection (Fig. 7b), the steroid concentrations are still above CCβ levels. Due to the second injection at Day 7 and the third injection at Day 14, the concentrations of the free steroids are higher than the 't=0 concentration' even after Day 6. It has to be mentioned that the elimination time is defined as the time it takes to reach the concentration of less than or equal to CCβ or to reach the 't=0 concentration' in case of a natural occurring background for example for 17α-estradiol and/or 17α-testosterone.

Regarding the steroid elimination time after the pour-on application, it is concluded that the CCβ or 't=0 concentrations' are reached within 5 days after the first application. No increase of steroid concentration is monitored after the second and third applications. The 17α-testosterone concentrations measured at day 42 and day 98 in Fig. 8a are probably outliers due to some interfering compounds. The results are not confirmed in Fig. 8b. It is possible that there is an increase of free steroid concentrations after the second and third applications (Fig. 8b) but due to the sample collection scheme, the possible increases were not monitored. The increase of free steroid concentration is expected in the samples collected 1–3 days after application; in this study, in the samples of Day 8, 9 and 10 and Day 15, 16 and 17, but, unfortunately, no samples were collected at these specific times.

From pharmokinetic experiments, it is known that the steroid esters when entering the bloodstream are hydrolysed to the free steroids viz. 17β-testosterone and 17β-estradiol. Furthermore, before elimination, free steroids are epimerized to α-testosterone and α-estradiol. C17 epimerization is a major pathway for steroids. This is also confirmed by the elimination kinetic described by Pinel [33]. In [33], it is demonstrated that the main metabolites detected in urine after i.m. injection of 17β-estradiol benzoate and 17β-nortestosterone laureate are 17α-estradiol and 17α-nortestosterone.

From the results obtained for the concentration of the free steroids in the present study, it is concluded that after i.m. injection, high concentrations of β-steroid esters enter the blood and are quickly hydrolysed to their free form—17β-testosterone and17β-estradiol—and after epimerization to the free 17α-steroids. However, after pour-on application, the concentrations of free β-steroids are much lower probably due to the fact that not all steroid esters enter the blood. It is possible that after pour-on application, the transport of the steroid esters to the hair is by another route than by blood transport, e.g. other extravascular or interstitial fluids [26]. So after pour-on application, the steroid esters are already hydrolysed, epimerized and eliminated before entering the blood resulting in much lower concentrations of the free β-steroids. After i.m. injection, the steroid esters enters the blood very quickly are hydrolyzed to the free β-steroids which are detected in the first 5 days after injection.

Conclusions

From the animal trial performed by i.m injection and pour-on application of steroid esters using different carrier solvents, it is concluded that: no significant differences are observed between the elimination rate of steroid esters in hair after i.m. injection and pour-on application of steroid esters. For both ways of application, the concentrations of steroid esters reach the CCβ levels (5–20 μg/kg) 5–7 weeks after single treatment and 9–11 weeks after repeated (three times) treatment. The identity of the carrier solvent during pour-on application does not have significant influence on the final steroid ester concentration in hair.

Even though high concentrations of steroid esters are measured in hair, no detectable concentration of steroid esters (<1 μg/l) were measured in the plasma of the treated animal. This is probably due to the quick hydrolysis of steroid esters to free steroids.

The only difference in i.m. injection and pour-on application measured in this study is the concentration of free β-form steroids in plasma. The concentration of free β-form steroids measured in plasma are much higher (ten times reaching 2,000 ng/l) after injection than after pour-on treatment. The concentrations of the α-form steroids are not significantly different. From these results, it is supposed that after the pour-on treatment, the transport of steroid esters to the hair is not only by blood but also by other

fluids. In this way, steroid esters are already hydrolysed, epimerized and eliminated before reaching the blood resulting in low concentration of the free steroids in plasma after pour-on application.

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Appendix V

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Review

The use of *omic* technologies for biomarker development to trace functions of anabolic agents

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ABSTRACT

The combat against misuse of growth promoting agents is a major topic in agricultural meat production and human sports. In routine screening, hormone residues of all known growth promoting agents are detected by immuno assays or chromatographical methods in combination with mass spectrometry. To overcome the detection by these routine screening methods new xenobiotic growth promoters and new ways of application were developed, e.g. the combination of different agents in hormone cocktails are employed. To enable an efficient tracing of misused anabolic substances it is necessary to develop new screening technologies for a broad range of illegal drugs including newly designed xenobiotic anabolic agents. The use of *omic* technologies like, transcriptomics, proteomics or metabolomics is a promising approach to discover the misuse of anabolic hormones by indirectly detecting their physiological action. With the help of biostatistical tools it is possible to extract the quested information from the data sets retrieved from the *omic* technologies. This review describes the potential of these *omic* technologies for the development of such new screening methods and presents recent literature in this field.

Contents

1. Introduction

Natural steroid hormones are synthesized from cholesterol and they are strongly involved in endocrine and paracrine regulation of growth and differentiation in most tissues. Some steroid hormones, like estradiol or testosterone show anabolic functions by enhancing body protein accretion and mobilizing fat stores, which results in an increased growth rate [1]. These properties are deep-rooted in the evolution of vertebrates. The sex steroids testosterone and estradiol have effects on behavioral, morphological and physiological traits. Estrogens stimulate protein- and mineral retention during pregnancy which is important for the development of the embryo. Testosterone promotes sexual behaviors like courtship, and improves growth of skeletal muscle which is important for defending the territory [1,2].

Steroid hormones participate in the establishment of muscle tissue and bone density. After menopause women and also older men often suffer from a loss in muscle mass (sarcopenia) and bone mineral density (osteoporosis) which may lead to frailty [3–6]. Both conditions are related to the decrease in the endogenous production of anabolic sex hormones, mainly estradiol and testosterone [5]. Men and women suffering from frailty are treated with

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testosterone or estradiol but both therapies are associated with negative side effects like skin virilization in women, prostate hypertrophy in men and an increased risk of cancer [7–9]. An alternative to the treatment with natural testosterone or estradiol are synthetic molecules called SARM (selective androgen receptor modulators) and SERM (selective estrogen receptor modulators), which bind to the steroid hormone receptors exhibiting predominantly tissue selective effects [10].

In human sports and agricultural meat producing animals the myotropic, growth promoting properties of steroid hormones are very beneficial. Used orally, the natural steroid hormones testosterone and estradiol are almost inactive. Besides these natural steroids the xenobiotic hormones trenbolone acetate (TBA), zeranol and melengestrol acetate (MGA) were developed by US companies to be used as anabolics in food producing animals. Whereas only MGA is orally active, the other drugs have to be applied by implantation [11].

Besides steroid hormones, the substance group of β-agonists has also been used as growth promoter in animal husbandry and human sports. β-agonists are well known in medicine due to their vasodilative attributes to treat asthma and other pulmonary diseases [12]. A widely spread drug for this application is salbutamol, which is given by the inhaled route to act directly on the smooth muscle cells in the bronchia. Synthetic β-agonists like salbutamol or the orally active clenbuterol are derivates of the adrenal medulary hormone epinephrin and the neurotransmitter norepinephrin, which are the natural agonists of the β-adrenergic receptor [13].

Several studies document the anabolic action of β-agonists in farm animals and also in laboratory animals. The daily weight gain of bulls treated with β-agonists was shown to be significantly higher than that in the untreated control group [14]. Increased growth rates and improved feed conversion could be observed in finishing bulls fed climaterol [15]. Rats fed clenbuterol improved live weight gain and feed efficiency as well as increased muscle mass due to hypertrophy of muscle fibres [16]. Another effect of β-agonists is the degradation of fat stores and hence the increase of the fat to lean meat ratio [17]. Because of that impact besides anabolic steroid hormones, these substances are used as growth promoters in animal husbandry [1,18,19]. Zilpaterol and ractopamine were developed by international companies to modify nutrient partition in food producing animals.

In meat production growth promoters are used to increase productivity and to reduce costs by improving weight gain and feed efficiency [20,21]. The use of growth promoters is approved in some countries like the USA, Canada, Mexico, Australia and South Africa. It has been proven that hormone residues in meat are increased and have adverse side effects to the consumer [20,22–24]. Therefore the use of anabolic agents in meat producing animals and also the import of meat derived from cattle given these substances is forbidden in the EU since 1988 (88/146/EEC). To enforce the EU-directive, permanent surveillance is essential [1,20–22,25,26].

In human sports, the application of anabolic substances to increase muscle performance, called doping, increased in the past 40 years [27–31]. Anabolic agents are not only used by competitive athletes, professional body builders or weight lifters, but more and more by amateurs to improve appearance and body shaping [32]. The World Anti-Doping Agency (WADA) yearly publishes a list of drugs and substance classes that are forbidden to be taken during training and competition [11,32,33]. The large number of doping cases during the Tour de France 2007 showed the importance of improving the screening techniques that can be used in future doping control practice and the requirement to develop new approaches to become more efficient in view of the upcoming new classes of growth promoters.

To uncover the abuse of anabolic agents in animal husbandry and human sports hormone residues are detected using immuno assays or chromatographical methods in combination with mass spectrometry [34–37]. With these methods only known substances can be discovered. To enable an efficient tracing of misused anabolic substances it is necessary to develop new technologies to screen for a broad range of illegal drugs including newly designed xenobiotic anabolic agents.

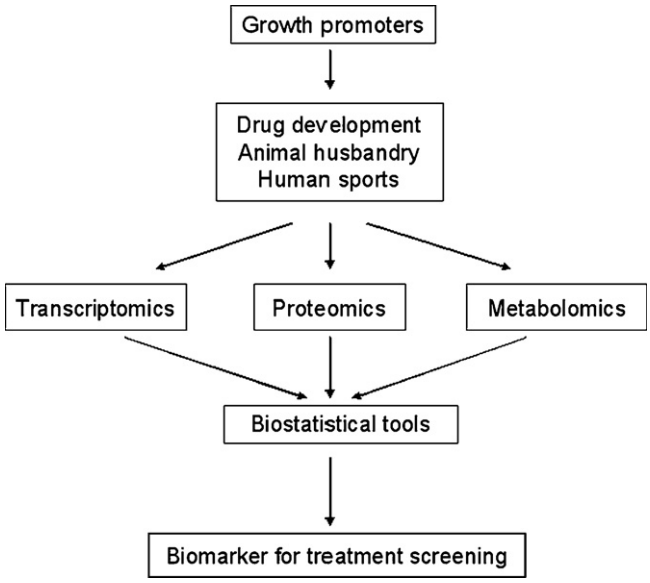

Fig. 1. Scheme of the use of *omic* technologies to trace anabolic hormone functions.

In molecular medicine, e.g. in cancer research, the development of molecular biomarkers is already a common approach in diagnostics. Plasma biomarkers are developed for prognostic use and tumor biomarkers are used to develop treatment strategies for each individual patient [38,39]. To develop such biomarkers *omic* technologies, like transcriptomics, proteomics and metabolomics are applied [39–41].

The use of such *omic* technologies will be a promising way to develop a biomarker pattern based on physiological changes that are caused after illegal application of anabolic agents (Fig. 1).

This review reflects efforts made during the last two decades in the field of screening for anabolic agents in animal husbandry and describes physiological and molecular effects of anabolic agents on different tissues in order to illustrate the potential of *omic* technologies for the development of reliable molecular biomarkers for anabolic agents. Literature research was done by using common databases for biomedical literature.

2. Molecular mechanisms of steroid hormone signaling

Steroid hormone receptors belong to the family of nuclear receptors and show a high affinity to their corresponding hormone [42,43]. They are either localized in the cytoplasm moving to the cell nucleus upon activation or directly in the nucleus waiting for the steroid hormones or active analoga to enter the nucleus and activate them [44]. Steroid receptors consist of different domains like a DNA binding domain, a nuclear localization domain, a ligand binding domain and several transactivation domains [42]. Without a bound ligand the steroid receptors exist as a steroid receptor complex, associated with different heat shock proteins (hsp90, hsp 56, hsp70) and p23 [45–47]. Binding of the ligand results in a conformational change which leads to the dissociation of the HSP-complex from the receptor. After dimerization the receptor binds to specific sequences in the promoter region of steroid hormone regulated genes, called hormone responsive elements (HRE) [42,43,48]. After DNA binding, different coregulators that are needed for

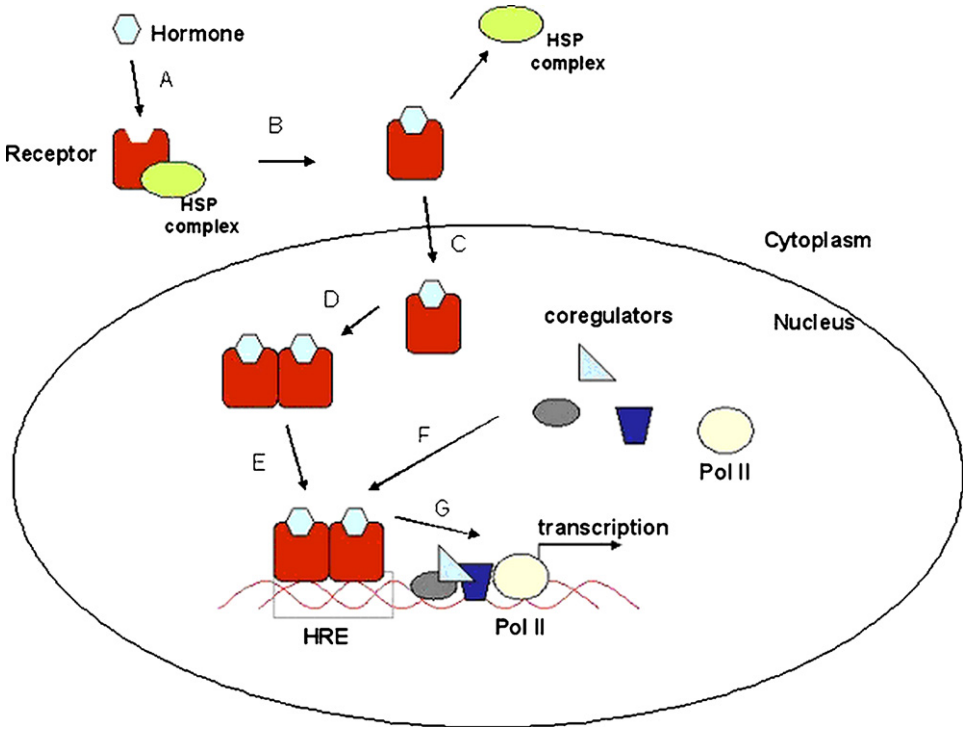

Fig. 2. Schematic diagram of the activation of a cytoplasmic steroid hormone receptor. After hormone binding (A) the HSP complex dissociates from the receptor (B), the hormone receptor complex translocates to the nucleus (C), dimerizes (D) and binds to a hormone responsive element (HRE) in the promoter region of a specific gene (E). After binding to the HRE different coregulators of transcription are recruited (F), which are responsible for transcriptional activation [49,50].

transcriptional activation are recruited. These coregulators have different functions. They either enhance or repress transcription through enzymatic activities like acetylation, deacetylation, kinase activity or methylation [49]. These coregulators are for example responsible for chromatin remodeling or the recruitment of RNA polymerase II (Pol II) [50] (Fig. 2). Another possibility of regulating gene transcription by steroid hormones is to influence or recruit other transcription factors like AP1 [51,52] or NFκB [53].

Steroid hormones not solely regulate gene transcription activity but also influence the stability of generated mRNA. They are able to stabilize or destabilize specific mRNA. Most is known about the influence of steroid hormones on the stability of their receptor mRNA. Whereas steroid receptor protein is normally down-regulated by their ligands, the regulation of the stability of steroid receptor mRNA may be positive or negative. Regulation of mRNA stability is not restricted to steroid hormone receptors, other genes are also regulated by similar mechanism [54].

Sex steroid hormone receptor signaling is primarily important in tissues of the reproductive tract like uterus, ovary, vagina, testes or prostate. But also other tissues like muscles, liver, kidney, lung, spleen, blood cells and parts of the gastrointestinal tract express steroid hormone receptors and are influenced by steroid hormones [55–57].

3. Molecular mechanisms of β-agonist signaling

As β-adrenergic receptors are present on almost every mammalian cell their agonists exert diverse biochemical effects. β-adrenergic receptors belong to the group of seven-span trans membrane receptors. Physiological mechanisms of β-agonists are mediated by binding of the agonist to the β-adrenergic receptor and the following induction of a G-protein coupled signaling cascade (Fig. 3).

The α-subunit of the G-protein thereby activates the enzyme adenylate cyclase (AC), which produces cyclic adenosine monophosphate (cAMP) as intracellular signaling molecule. After binding to the regulatory subunit of protein kinase C (PK C) cAMP removes the catalytic subunit to enable the enzyme to phosphorylate several intracellular proteins. This phosphorylation can either activate (e.g. hormone sensitive lipase) or deactivate (e.g. acetyl-CoA-carboxylase) enzymes. PK C also phosphorylates cAMP responsive element binding protein (CREBP), which binds to cAMP responsive elements (CRE) in regulatory regions of genes to stimulate transcription [58,59].

4. The use of *omic* technologies for biomarker research

4.1. Transcriptomics

The transcriptome is the complete set of RNA transcripts present in a particular cell, and the most prominent candidates investigated in research are the messenger RNA (mRNA), micro-RNA (miRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Transcriptomics describes the global study of gene expression at a certain time point, for example as a reaction after a specific treatment.

Methods used nowadays for studying transcriptomics are cDNA hybridisation microarrays, conventional RT-PCR and quantitative real-time RT-PCR (qRT-PCR). Microarrays have the advantage that a whole set of genes can be analyzed on one array, but they are not sensitive enough to measure minimal changes in gene expression. Using quantitative RT-PCR genes can only be quantified separately, but this method is more sensitive, its dynamic range of quantitation is much wider, it is better reproducible and less expensive than microarray experiments. Another advantage of qRT-PCR is that more biological samples can be measured in one experiment [60,61].

The combination of both, finding biomarker candidate genes using microarrays or exploring the literature and verification of these changes in gene expression using qRT-PCR is a promising way to find gene expression biomarkers.

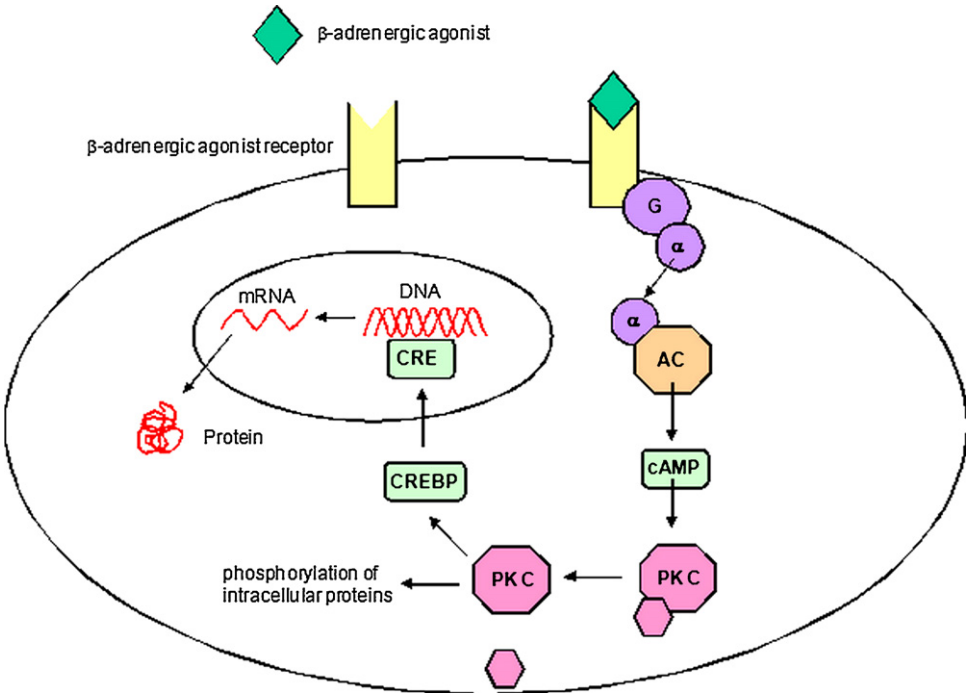

Fig. 3. Signaling cascade leading to the physiological mechanisms of β-adrenergic agonists.

The potential power of gene expression biomarkers for diagnostic use has already been demonstrated in cancer research [41,62–66]. Physiological changes can be quantified on the level of gene expression. Anabolic hormones have several physiological effects and therefore finding gene expression biomarkers could be a promising approach to develop a screening method for the use or misuse of anabolic hormones.

There are numerous reports that steroid hormones and also β-agonists affect gene expression in different organs. Reiter and coworkers [67] quantified changes in mRNA expression for a number of genes in bovine liver, muscle and uterus that are controlled by different xenobiotic anabolic agents and found several regulated genes that could be first candidates for developing gene expression biomarkers.

The influence of steroid hormones on the mRNA expression of several genes could be shown by some research groups in different tissues. Promising candidate genes for the development of a screening method in cattle are IGF-1 in liver and muscle [56,67–69], steroid hormone receptors in various tissues like liver, muscle, uterus, the gastrointestinal tract, kidney, prostate and blood cells [56,57,67,70,71], and various inflammatory, apoptotic and proliferative genes in blood cells [71–73]. β-agonists are known to affect mRNA expression of different muscle proteins like α-actin, myosin or calpastatin in cattle. The mRNA expression of β-adrenergic receptors are also known to be influenced by their ligands [74,75].

Most of these tissues can only be taken after slaughter and so they present no promising tissues for developing a doping screening method in humans. In humans only non-invasive sampling of blood, urine or hair could serve as matrix to find gene expression changes, because they can easily be taken from the individual.

In vivo studies in humans regarding gene expression changes caused by steroid hormones are rare, but various cell culture models exist. Studies in different human blood cell culture models suggest that steroid hormones alter gene expression in human blood cells [76–78]. An *in vivo* study on *macaca fascicularis* demonstrates that testosterone and the SARM LGD2941 influence the expression of apoptotic and proliferative genes in blood cells [79].

Reiter et al. [80] could monitor gene expression changes in cell culture experiments with human hair follicle dermal papilla cells that were treated with stanozolol. In another *in vivo* study, they could show that it is possible to extract RNA out of hair follicle cells and that gene expression in these cells is also influenced by steroid hormones [81]. As shown, blood and hair roots represent promising tissues to find gene expression biomarkers with potential to develop a non-invasive screening method based on gene expression patterns.

4.2. Proteomics

The term proteomics describes the study of the proteome which is the actual content of all proteins present in a cell, tissue or organism at a specific physiological stage or as a reaction to a certain treatment.

The use of proteomics for biomarker screening is already common in clinical diagnosis and research. In the diagnosis of different diseases or physiological states blood protein biomarkers are routinely used. Troponins for example are indicators for heart attack, alkaline phosphatase for biliary problems and human chorionic gonadotropin (hCG) is the ultimative marker for early pregnancy [82,83].

In cancer research, as malignant transformation and clonal proliferation of altered cells go in line with alterations in protein expression, proteomics can be used for diagnostic purpose and early detection of cancer [83,84].

Advanced methods for proteomic investigations include two-dimensional gel electrophoresis (2D-gel), mass spectrometry and protein microarrays [38,41] which can be used for biomarker research. These methods are suitable to screen for all multisided changes in protein expression that are caused by a changed physiological status or induced by a specific treatment e.g. by anabolic steroid hormones. This way of biomarker screening can be named as "*de novo*" approach [82,85] with the advantage, that numerous proteomic changes, also those that are so far unknown can be evaluated. Another way of screening for biomarkers is the evaluation of candidate proteins by screening the actual literature or by regarding

physiological effects that are present at a specific physiological state or are induced by a certain treatment [82,85]. Methods that can be used for this approach are 1-D electrophoresis, RIA (radio immuno assay), ELISA (enzyme linked immuno sorbant assay) or western blot. The advantage of these methods is that only a numerable number of proteins have to be analyzed, immuno assays are more sensitive and the analysis of data sets is well arranged.

Regarding known effects of steroid hormones on protein expression or excretion it could be investigated if the candidate protein approach will be a promising way for developing a potential screening method for the application of anabolic agents.

Very promising proteins for developing a protein biomarker pattern will be IGF-1, IGF-1BP3 and somatotropin (ST). Numerous reports showed these proteins to be increased after the use of anabolic agents in blood plasma of animals and humans [86–93]. Clenbuterol has been shown to down regulate the beta adrenergic receptor and the glucocorticoid receptor in blood cells of veal calves. Dexamethasone also down regulates plasma levels of the glucocorticoid receptor in calves [94]. Different lipoproteins or apolipoproteins are also affected by anabolic hormones in cattle and humans. Hartgens and coworkers [95] could show that androgenic anabolic hormones (AAS) increase plasma protein levels of low density lipoprotein cholesterol and apolipoprotein B and decrease protein levels of high density lipoprotein cholesterol, apolipoprotein A1 and lipoprotein(a) in athletes. An increase of apolipoprotein A1 in plasma of calves by the xenobiotic androgen boldone was demonstrated by Draisci et al. [96]. Propeptide of type III procollagen is known to be a potential marker for the use of anabolic agents in humans [97]. In female calves treated with a combination of oestradiol plus nortestosterone the content of propeptide of type III procollagen is also increased [98]. In the same animal trial it was shown that the combination of nortestosterone plus oestradiol decreases plasma ir-inhibin levels in male calves and that treatment with dexamethasone decreases plasma osteocalcin in veal calves independent of gender [99]. Gardini et al. [100] tried to evaluate protein biomarkers for the treatment of calves with an anabolic combination of estradiol-17β, clenbuterol and dexametasone by using the combination of 2D-gel and mass spectrometry. They found two regulated proteins in liver tissue (up-regulation of reticulocalbin, down-regulation of adenosine kinase) which could be possible new biomarker candidates for the treatment with this drug combination [100,101].

Apoptotic factors and pro- and anti-inflammatory factors are also promising biomarker candidates because of the known effects of anabolic steroid hormones on apoptosis in different tissues [102–104] and the immune response, in which estrogens show pro-inflammatory and androgens anti-inflammatory effects [105].

Although the proteomic approach is a very promising way to develop a biomarker screening pattern, but up to now very few publications are available in the open literature.

4.3. Metabolomics

The metabolome is the collectivity of small-molecule nutrients and metabolites (e.g. metabolic intermediates) in a biological sample. The term metabolomics (also metabonomics) has been established in analogy to transcriptomics and proteomics and describes the study of the metabolome at a certain time point.

Other than transcriptomics and proteomics there is no preferential technique for metabolic investigations so far. In former times changes in the metabolome were measured by detecting single metabolites or degradation products of the certain metabolic pathways in body fluids like urine or blood by chromatograpical or kinetic methods (e.g. Jaffé reaction for the detection of creatinine). The concentration of metabolites like glucose or fatty acids could also be determined by enzymatic methods (e.g. glucose oxidase method) or colorimetric methods using commercially available kits [106,107]. Nowadays due to the technological developments and the availability of hundreds of different standards it is possible to simultaneously measure a great number of substances in one assay to reflect the metabolic status of a certain cell. This metabolic screening method is used for biomarker development mainly in research fields concerning cancer or other diseases using gas or liquid chromatography coupled with mass spectrometry and NMR spectroscopy [108–111]. Although there are few efforts by now, these technologies could also be applied for metabolomic studies in the investigation of hormone function in the organism.

The anabolic effects of β-agonists are mainly due to an increase in muscle protein deposition and a decrease in fat accretion [112]. Metabolites that are involved in these mechanisms could act as potential biomarkers for the use of β-agonists. Creatinine, an indicator for muscle protein synthesis, and Nτ-methylhistidine (MH), an indicator for muscle protein degradation, act as metabolic indicators for protein metabolism [113,114]. Williams et al. [114] found a higher creatinine excretion and a reduction of MH elimination in the urine of animals fed with clenbuterol compared to the control group.

The decrease in body fat due to the application of β-agonists can be explained by an induction of lipolysis and an inhibition of lipogenesis. Higher concentrations of non-esterified fatty acids (NEFA) in the plasma of animals treated with β-agonists occurred in several studies [106,107,115,116]. Not only the plasma concentration of NEFA, but also the fatty acid composition in the plasma was shown to be changed by clenbuterol [107].

Various studies showed an increase in the plasma glucose levels due to enhanced gluconeogenesis and glycogenolysis in the liver and the breakdown of muscle glycogen to supply the energy sources for the formation of muscle protein after the administration of β-agonists [107,115–118]. Natural catecholamines have been shown to exert indirect mechanisms on the release of several hormones, e.g. the inhibition of the insulin release and thereby the insulin-mediated glycolysis and glycogenesis [118]. In contrast, under the influence of synthetic β-agonists an increase in the insulin level could be observed [112,119].

As concurrently an increase in the glucose, lactate and NEFA plasma levels occurs the development of an insulin resistance under the treatment is suggested. The release of energy substrates goes along with an increase in the blood flow to alleviate the transport to the target tissues [106,115,120]. Equally large amounts of lactate occur in the plasma suggesting an increased glycolysis in muscle tissue for the formation of ATP as energy source for the development of muscle mass [112,116,117].

The effects of β-agonists are mainly transient and the initially marked response becomes attenuated due to a lower responsiveness and a down-regulation of β-adrenergic receptors [120,121].

There have also been efforts to detect perturbations in the metabolic profile after the administration of steroid hormones to reveal the illict application as growth promoters. Blood metabolites like creatinine or creatine kinase, which are associated with muscle function, or the plasma urea levels, which are known to be an early indicator for anabolic effects in cattle, could serve as potential biomarkers for treatment screening. Mooney et al. [98] measured this metabolites by UV based enzymatic and kinetic methods and showed a significant increase in the plasma urea levels under the influence of estradiol-17β benzoate plus nortestosterone decanoate, but no alteration in the creatinine levels or the creatine kinase activity compared to the control animals.

Cunningham et al. [122] investigated different blood metbolites of ruminants treated with anabolic steroids by standard blood chemistry analysis to investigate if these parameters could be used in a screening test to detect illegal use of growth promoting hormones. Herein no significant change in the urea levels between

treated and untreated animals, but a significant increase in the creatine levels of heifers treated with nortestosterone decanoate and steers treated with estradiol benzoate was demonstrated. Also substance specific effects on the billirubin levels were shown, with levels being increased in steers and decreased in heifers. As these effects just occur on certain days and not over the complete course of the study period these parameter could not serve as marker for anabolic treatment [122].

In human sports the investigation of the steroid profile is used as a versatile screening tool for routine doping control. The steroid pattern in urine shows distinct ratios of several endogenously synthesized steroid hormones due to the natural excretion. These ratios can be perturbated by increasing or decreasing certain steroid concentrations during the administration of exogenous anabolic compounds [123]. As these ratios can also be altered by natural reasons like the belonging to different ethnic groups this measurement is not sufficient to prove a doping suspicion. GC-C-IRMS can be used to reveal the origin of the applied substance as exogenously applied and endogenously synthesized steroids vary in the ratio of the carbon isotopes ^{12}C and ^{13}C. Pharmaceutical steroids show lower amounts of ^{13}C, as they are not synthesized de novo, but derived from plant materials [124].

However compared to the β-adrenergic agonists few is known about the metabolic effects of steroid hormones and more targeted investigation have to be done to make a statement on changes in body fluids.

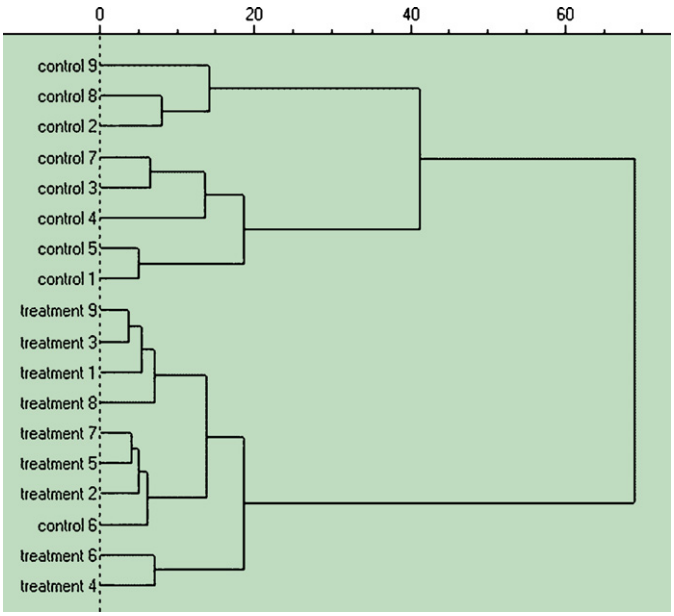

Fig. 4. Cluster dendogram of a qRT-PCR experiment with hormone treated and untreated heifers. Quantitative RT-PCR expression results of vaginal epithelial cells of untreated (control) and treated (treatment) heifers were clustered using GenEx version 4.3.6 Software (MultiD Analyses AB, Gothenburg, Sweden).

5. Bioinformatics

Regardless of which *omic* technology is used for biomarker research, bioinformatical tools are necessary to extract the needed information from the resulting data set.

There are very few examples of unequivocal evidence given by a single biomarker like the trophoblast marker hCG being only present in early pregnancy. In biomarker research the scientist gets a pattern of biomarkers with multiple factors being influenced quantitatively by the drug or the specific physiological stage. The most important question is how do deal with a huge data set to extract, interpret and visualize the intended information. To transform the high-dimensional data into a reduced subspace for representing data in far fewer dimensions, methods for dimensionality reduction are needed [125]. In combination with pattern recognition technologies the identification and visualization of the desired information is approached.

A simple method to classify samples by genomic, proteomic or metabolomic expression patterns is two or three dimensional scatter plot [61]. Using this method only two or three transcripts, proteins or metabolites can be included [60,61]. If more components should be taken into account, multivariate analysis methods are required. Principle components analysis (PCA) reduces multidimensional data sets to lower dimensions called "principle components" [60,61,126]. Each analyzed sample will be visualized by one spot that results from diminishing all collected data of the specific sample to two principle components and so each analyzed sample will be represented by one spot. Employing this method for growth promoter treatment screening will ideally result in a graph with two groups of spots. One group representing the untreated controls and the other group representing the treated individuals. PCA was effectively used by Riedmaier et al. [71] to identify potential gene expression biomarker patterns for anabolic treatment in bovine blood.

To combine the results of two *omic* technologies canonical correlation analysis (CCA) can be used. This method summarizes the relationship between two sets of variables and shows what is common amongst the two sets [126]. To combine the results of two or more *omic* technologies linear or multiple discriminant analysis (LDA or MDA) can be used [126]. Based on a set of measurable features, these methods classify objects into groups. Screening for the use of anabolic hormones by CCA, LDA or MDA should result in a picture similar to those of PCA, where the treated individuals are separated from the untreated controls.

Another method for visualizing treatment patterns based on multivariate data is hierarchical cluster analysis. The hierarchical order is represented by a tree dendogram in which related samples are more closely together than samples that are more different [60,126]. Used in anabolic treatment screening hierarchical cluster analysis should result in a tree where the treated or the untreated samples respectively are close together and the group of treated samples is separated from the group of untreated samples. Fig. 4 shows a dendogram of qRT-PCR data received from vaginal epithelial cells of heifers, treated with anabolic hormones (unpublished data). The treated ($n = 9$) and untreated ($n = 9$) individuals are close together.

Regardless of which biostatistical method will be employed for treatment screening, it is always necessary to have a high number of untreated controls serving as basis for physiological normal individuals. High biological variance between each individual are caused by genetically diversity or environmental conditions [127]. To deal with these differences between various individuals a high number of control samples representing the investigated group of animals of humans is needed.

In summary with advanced biostatistical method marking and reliable classification of treated animals is possible.

6. Conclusions

The use of *omic* technologies will be a promising way to develop new screening methods for the detection of the misuse of anabolic steroids and β-agonists based on the physiological changes caused by these substances. Very sensitive methods, like quantitative RT-PCR and mass spectrometry allow the quantification of very small changes in gene expression, protein expression or in the presence of metabolites. With the help of biostatistical tools it is possible to extract the quested information from the resulting data sets.

The discovery of newly designed substances, new modes of drug misuse or other kinds of manipulation in animal husbandry or

sports – like erythropoietin, blood or gene doping – will be a future challenge to *omic* techniques.

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Appendix VI

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PAPER

Changes in the miRNA profile under the influence of anabolic steroids in bovine liver

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miRNAs are regulatory RNA molecules. The analytical interest rose over the past 10 years especially in clinical diagnostics as miRNAs show specific expression patterns in several human diseases like diabetes or cancer. Therefore, it is expected that miRNA profiles might be used as biomarkers in early diagnosis. The idea of establishing biomarkers is also present in veterinary drug analysis, *e.g.* in the surveillance of illegal use of anabolics. Transcriptomics is a promising approach in the detection of anabolics misuse. However, miRNA expression patterns have shown their superiority over mRNA patterns in clinical diagnostics. Thus, the influence of anabolic steroids on miRNA expression in bovine liver should be investigated and an expression pattern should be validated, which might be used as a treatment biomarker. An animal experiment was conducted with 18 heifers equally allocated to a control and a treatment group, which was implanted with TBA plus E2. Liver samples were screened for miRNA expression using PCR arrays. Expression of 11 prominent miRNAs was validated *via* single assay qPCR. Herein, the following expression pattern could be found with an up-regulation of miR-29c and miR-103 and a down-regulation of miR-34a, miR-181c, miR-20a and miR-15a ($p < 0.05$ each). Using principal components analysis (PCA), the control group could clearly be distinguished from the treatment group, when integrating gene expression results from both miRNA and mRNA. So, the combination of different transcribed targets (mRNA plus miRNA) might be a promising approach to find a valid expression pattern to be used for anabolic treatment screening.

Introduction

MicroRNAs (miRNAs) are a novel class of non-coding small RNA molecules with a length of approximately 22 bp, which exhibit regulatory functions on gene expression. miRNAs participate in the post-transcriptional regulation of a variety of important physiological processes like proliferation, apoptosis and differentiation by inhibition of translation or degradation of mRNAs. The first miRNAs, lin-4 and let-7, were described in *Caenorhabditis elegans* already in 1993,[1] but the name "miRNA" primary alluded in 2001. The interest in valid miRNA expression analysis rose over the past ten years especially in the medical diagnostic fields. Herein, miRNAs have been shown to be dysregulated in different pathological events in humans. The miR-29 family has been shown to be implicated in the genesis of diabetes. Besides other diseases (*e.g.* diabetes, polycystic liver disease), miRNAs are implicated in the pathogenesis of different cancer types (lung, liver, breast, prostate, gastrointestinal tract).[2–7] Herein, miR-130a, miR-34a, miR-106 and the miR-29 family are known to be dysregulated in hepatocellular carcinoma (HCC),[7,8] which is also a known long-term side effect on anabolic misuse. In cancer diagnosis, miRNA profiles have proven their superiority over mRNA profiles as miRNA patterns seem to be generated in a tissue- and disease-specific manner, which is not the case for other established biomarkers.[9–11] Furthermore, after the transcriptional processing (pri-miRNA, pre-miRNA), there are no further modifications in the mature miRNAs and thereby no different variants *e.g.* splice variants of the same molecule facilitating an accurate detection.[12] Due to these facts, specific miRNA expression patterns might be auspicious to be used as clinical markers in diagnostics.

The idea of establishing biomarkers may also be realized in veterinary drug analysis. Various groups are doing research on the discovery of biomarkers for the surveillance of misuse of illegal growth promoters like anabolic steroids using omics-technologies.[13–15] Herein, transcriptomics might be the method of choice, as steroid hormones are known to influence gene expression in many primary and secondary dependent tissues in the organism. The potential of this approach as a method to evaluate biomarkers for anabolic treatment has been proven in several studies.[13,16] The actual study focused on liver as it is an important organ in steroid metabolism and it has been shown to be a promising target organ for biomarker development.[34] In

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liver, miRNAs have been demonstrated to play an important role in a variety of biochemical processes like proliferation, apoptosis and glucose metabolism,[17,18] which are also known to be targeted by anabolics.[34] There also exist known E2-influenced miRNAs expressed in the liver like miR-27b, miR-103 and miR-98.[19] So possibly, the expression of miRNAs could be influenced by the application of exogenous hormones. Thereby, the investigation of miRNA profiles might be an innovative and precise method to trace anabolic treatment.

Revalor H containing trenbolone acetate (TBA) plus estradiol-17β (E2) was chosen for treatment in the actual study as it is an anabolic supplement widely used and licensed in several countries including the Republic of South Africa. Several investigations showed its efficacy in cattle and the beneficial effect on meat quantity and composition.[20] Also, the activity of this combination on day 42 has been proved, as the intended effect is persistent up to day 105 after implantation and residues of the applied drug are detectable in the blood at least up to day 90.[21]

In the current study, changes in the miRNA expression profile under the influence of anabolic steroids should be investigated for the first time in bovine liver. The application of appropriate biostatistical methods should help to extract the required information from the measured miRNA expression patterns.[22] Herewith it should be attempted to find a gene expression pattern (mRNA and miRNA), which might be used as a biomarker for the surveillance of anabolics misuse.

Experimental

Experimental design

18 healthy, non-pregnant, 2-year old Nguni heifers were divided into a control and a treatment group of 9 animals each. Animal attendance was done by the Onderstepoort Veterinary Institute (Onderstepoort, Pretoria, South Africa). The treatment group was implanted with the anabolic preparation Revalor H (Intervet, Spartan, South Africa) containing 140 mg TBA plus 20 mg E2. Implantation was done according to the instructions into the middle third of the pinna of the ear. At slaughter after 42 days of treatment, liver samples were collected. The tissue was removed in less than 15 min, conserved in RNA later (Applied Biosystems, Darmstadt, Germany) immediately after the removal and further stored at −80 °C.

Extraction

Total RNA has been extracted with the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

RNA concentration measurement

Total RNA concentration was measured spectrophotometrically using the NanoDrop (PeqLab Biotechnologie GmbH, Erlangen, Germany). $OD_{260/230}$ and $OD_{260/280}$ were checked concerning RNA purity.

Analysis of RNA integrity

Intactness of RNA samples has been proven to be important for mRNA as well as miRNA expression studies.[23,24] RNA integrity control was performed with the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) using the Eukaryote Total RNA Nano Assay (Agilent Technologies). The RNA integrity number (RIN) served as the RNA integrity parameter. The RIN is calculated based on a numerical system from 1 to 10, with 1 being most degraded and 10 being most intact.[25]

miRNA expression profiling

miRNA expression profiling was done using human miRCURY Ready-To-Use PCR Panels (Exiqon, Vedbaek, Denmark) measuring 730 different miRNAs on two plates. For miRNA expression screening, 3 samples of the control and the treatment group, respectively, were used.

For cDNA synthesis from miRNA, samples were poly-adenylated, elongated and reverse transcribed using the miRCURY Universal cDNA synthesis Kit (Exiqon). A sample amount of 25 ng was diluted to a final volume of 14 μL, which were added to 4 μL 5× reaction buffer plus 2 μL enzyme mix. In the Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany), the reaction mix was incubated at 42 °C for 60 min followed by a heat-inactivation of the enzyme at 95 °C for 5 min. 2180 μL of nuclease free water (Exiqon) were added to the cDNA from the reverse transcription (RT) step to a final 110× dilution. This dilution was mixed 1 : 1 with 2200 μL of the miRCURY SYBR Green Mastermix (Exiqon). 10 μL of the final mastermix were added to every well on the Ready-To-Use Panels. For automation, pipetting was done with the epMotion 5075 LH (Eppendorf). After sealing with the 4s2 Thermal Sealer (4titude, Berlin, Germany), the plate was inserted in the Bio-Rad CFX Cycler (Bio-Rad, Munich, Germany) and the following temperature protocol was started: polymerase activation/denaturation step (95 °C, 10 min), cycling program (95 °C, 10 s; 60 °C, 1 min; ramp rate of cooling from 95 °C to 60 °C: 1.6 °C s^{-1}) and melting curve analysis.

cDNA synthesis for single assay quantitative PCR (qPCR)

miRNA samples were poly-adenylated, elongated and reverse transcribed using the miScript system (Qiagen). For a final volume of 10 μL, 2 μL 5× miScript buffer, 1 μL miScript reverse transcriptase and 2 μL RNase free water were mixed with 500 ng (100 ng μL^{-1}) of total RNA. The reaction was pipette in a 96-well plate and the plate was inserted in the Eppendorf Gradient Cycler (Eppendorf) and the here stated temperature protocol was started: 37 °C, 60 min; 95 °C, 5 min; 4 °C hold. After RT, all samples were diluted 1 : 6 to a final volume of 60 μL.

Validation of PCR array results with single assay qPCR

Single assay qPCR for miRNA was done using the miScript system (Qiagen) according to the manufacturer's recommendation. miScript Primer Assays for specific miRNA targets were synthesized and ordered at Qiagen. As no specific bovine primer assays are available, human assays were used after checking the sequence homology using http://www.mirbase.org/. 100% homology could be proven for all validated targets.

The qPCR mastermix was prepared with all necessary components as follows: 10 μL 2× QuantiTect SYBR Green PCR mastermix, 2 μL 10× universal primer, 2 μL 10× miScript primer

assay, and 4 μL RNAse free water. 18 μL of the prepared mastermix were mixed with 2 μL template from miRNA RT for a total volume of 20 μL. The following PCR protocol was started in the Eppendorf Realplex (Eppendorf): denaturation step (95 °C, 15 min), cycling program (95 °C, 15 s; 55 °C, 30 s; 70 °C, 30 s) and melting curve analysis.

Data evaluation

Gene expression data were analyzed using relative quantification. Data from miRNA expression profiling were initially processed using GenEx v. 5.0.2.8 (multiD Analyses AB, Gothenburg, Sweden). After merging data from all panels and interplate calibration, data were also subjected to relative quantification. Suitable reference genes (RG) for normalization of gene expression data were evaluated using the geNorm and Normfinder algorithm in GenEx v. 5.0.2.8 (multiD Analyses AB). These algorithms detect the most stably expressed genes in an experimental setup. Optimal number of RG was selected using pairwise variation analysis integrated in geNorm algorithm implanted in GenEx. miR-122 and miR-186 were used for normalization of PCR array as well as single assay PCR data. Data were normalized and relatively compared to the control group according to the $\Delta\Delta C_q$-model with the following formulas:[26]

$$\Delta C_q = C_{q(\text{target gene})} - C_{q(\text{reference gene index})}$$

$$\Delta\Delta C_q = \Delta C_{q(\text{treatment group})} - \text{mean } \Delta C_{q(\text{control group})}$$

The expression ratio of the treatment group compared to the control group is expected as $2^{-\Delta\Delta C_q}$ and represents the *x*-fold regulation with a value of 1 indicating no expression change after treatment.

The determined *p*-values of the statistical significance were examined using Student's *t*-test. Results with $p \leq 0.05$ were considered as statistically significant.

Linear regression was used to determine the correlation between C_q values from PCR arrays and single assay PCR. Significance of linear regression was analysed by Student's *t*-test by testing the slope to be different from zero. Level of significance was set for $p < 0.05$. Regression data were graphically plotted using SigmaPlot 11.0 (SSPS Inc, Chicago, IL, USA).

To visualize the multivariate response of the selected target genes to the treatment, the method of principal components analysis (PCA) was employed using GenEx v. 5.0.2.8 (multiD Analyses AB).[22] PCA involves a mathematical procedure that transforms a number of variables (here normalized expression values) into a smaller number of uncorrelated variables called principal components. By this, the dimensionality of the data is reduced to a number of dimensions that can be plotted in a scatter plot, here two dimensions. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible.

MIQE-Guidelines

This investigation was performed according to MIQE-Guidelines.[27]

Results

RNA integrity

All 18 samples were examined using the Eukaryote Total RNA Nano Assay on the Agilent 2100 Bioanalyzer. The RNA samples showed good RNA quality with a mean RIN of 8.34 ± 0.36, which is perfectly suitable for PCR analysis.[24]

Gene expression analysis

In the miRNA screening, 3 randomly picked samples from the control and the treatment group, respectively, were investigated ($n = 6$). Herein, 14 miRNAs were shown to be significantly up-regulated (Table 1) and 22 miRNAs were shown to be significantly down-regulated (Table 2) ($p < 0.05$ each). In further 14 miRNAs, a trend for regulation could be observed ($p < 0.1$), but these did not reach statistical significance.

The expression of prominent miRNAs, which might be candidates for physiological regulations, was validated in all experimental samples ($n = 18$) using single assay qPCR (Table 3). Significant down-regulations could be demonstrated in miR-15a ($p = 0.023$), miR-20a ($p = 0.024$), miR-34a ($p = 0.017$) and miR-181c ($p = 0.038$). miR-103 ($p = 0.040$) was significantly up-regulated. A trend for up-regulations could be shown for miR-29c ($p = 0.066$) and miR-106a ($p = 0.057$), in miR-320d a trend for down-regulation was observed, but no statistical significance could be proven for these data.

Results from single assay qPCR and PCR arrays have been correlated using linear regression to compare both methods (Fig. 1). Correlating the single values for the *x*-fold expression of the PCR array results with the single assay qPCR results from these 6 samples, which were investigated in both cases, a highly significant linear regression could be found showing good reproducibility of gene expression data gained from single assay or array qPCR.

Table 1 miRNAs shown to be up-regulated in bovine liver under the influence of TBA + E2 on the PCR array (* = $p < 0.05$; ** = $p < 0.01$; value 1.00 represents no expression change)

miR-	*x*-Fold regulation ($2^{-\Delta\Delta C_q}$)	*p*-Value	Significance level
412	3.18	0.006	**
103-2*	2.16	0.010	**
15a	1.23	0.010	**
192*	1.72	0.029	*
378*	22.04	0.030	*
532-5p	1.38	0.027	*
103	1.20	0.032	*
885-5p	1.50	0.030	*
let-7f2	1.23	0.032	*
152	1.62	0.041	*
493	1.97	0.036	*
744*	1.71	0.046	*
215	1.61	0.049	*
572	39.49	0.054	*
138	3.42	0.060	
192	1.49	0.061	
1247	1.79	0.066	
194	1.50	0.087	
505	1.52	0.094	
146-5p	1.27	0.090	
125a-3p	4.43	0.089	

Table 2 miRNAs shown to be down-regulated in bovine liver under the influence of TBA + E2 on the PCR array (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; value 1.00 represents no expression change)

miR-	*x*-Fold regulation ($2^{-\Delta\Delta C_q}$)	*p*-Value	Significance level
628-3p	0.15	0.001	***
130a	0.40	0.005	**
34a	0.31	0.006	**
29c	0.67	0.008	**
7-1*	0.57	0.016	*
29a	0.78	0.017	*
551b	0.32	0.017	*
765	0.29	0.018	*
132	0.56	0.022	*
29b	0.61	0.022	*
137	0.62	0.022	*
27b	0.53	0.022	*
98	0.74	0.026	*
181c	0.35	0.026	*
505*	0.05	0.027	*
708	0.41	0.033	*
455-5p	0.52	0.035	*
135a	0.37	0.037	*
486-5p	0.69	0.040	*
135a	0.36	0.040	*
433	0.40	0.046	*
518f	0.58	0.047	*
324-5p	0.82	0.057	
193-5p	0.73	0.065	
29c*	0.86	0.070	
126*	0.58	0.074	
93*	0.64	0.087	
494	0.57	0.089	
92a-1*	0.40	0.091	

Table 3 Validation of prominent miRNA candidates using single assay qPCR (* = $p < 0.05$; n.s. represents non-significant data; value 1.00 represents no expression change)

	x-Fold regulation ($2^{-\Delta\Delta C_q}$)			
miR-	↑	↓	*p*-Value	Significance
15a		0.49	0.023	*
20a		0.49	0.024	*
27b		0.97	0.58	n.s.
29c	1.30		0.066	n.s.
34a		0.67	0.017	*
103	1.30		0.040	*
106a	1.40		0.057	n.s.
138	1.74		0.2	n.s.
181c		0.75	0.038	*
320d		0.72	0.065	n.s.
433	1.15		0.89	n.s.

PCA of gene expression data has been produced as shown in Fig. 2 and 3. Animals from the control group are represented by grey squares, animals from the treatment group are represented by black circles. Gene expression data from regulated miRNAs from single assay PCR were plotted (Fig. 2) and herein, a slight separation could be observed between the control and the treatment group. This indicates that the measured miRNA pattern is not yet sufficient to differentiate between treated and non-treated animals. It might be possible to reach a better separation by integration of more target genes. Additionally, regulated

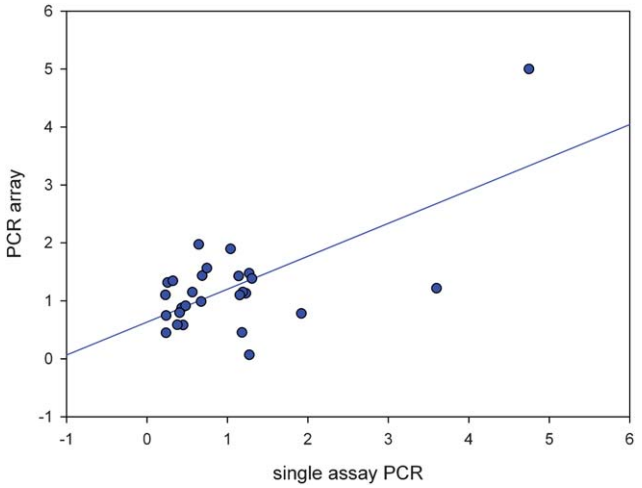

Fig. 1 Correlated *x*-fold regulation ($2^{-\Delta\Delta C_q}$) of the control group compared to the treatment group in the 11 candidate miRNAs measured both on the PCR array and validated in single assay PCR with the single assay PCR data on the *x*-axis and the PCR array data on the *y*-axis with a slope of 0.773 and $r = 0.662$ ($p < 0.001$).

miRNAs have been combined with regulated target genes from mRNA expression analysis from the same transcriptome (Fig. 3).[34] In this PCA, a clear differentiation between the animals from the control and treatment group could be observed with just one animal from the control group being expressed in a similar range as the animals of the treatment group.

Discussion

Few is known about the expression and functionality of miRNAs in bovine species making it impossible to gain sufficient information from the current scientific literature to define auspicious target miRNAs. Thus, a profiling method has to be applied to identify changes in the miRNA expression. Human ready-to-use PCR arrays containing 730 confirmed human miRNAs have been used as a screening method to find candidate miRNAs, as no species-specific screening methods for bovine are available,

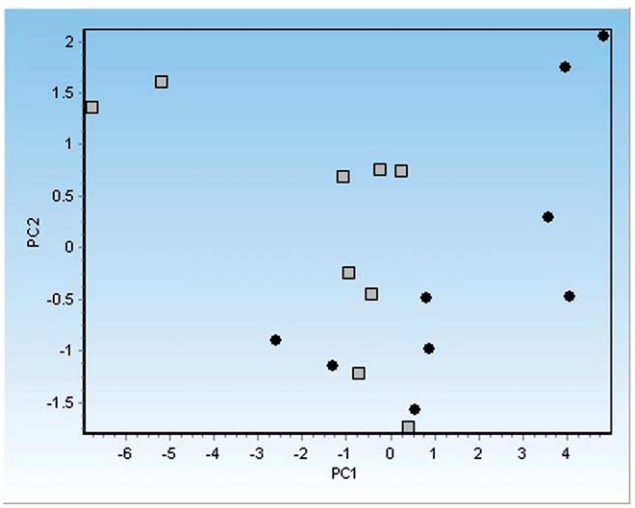

Fig. 2 PCA of 8 regulated target miRNAs from single assay PCR.

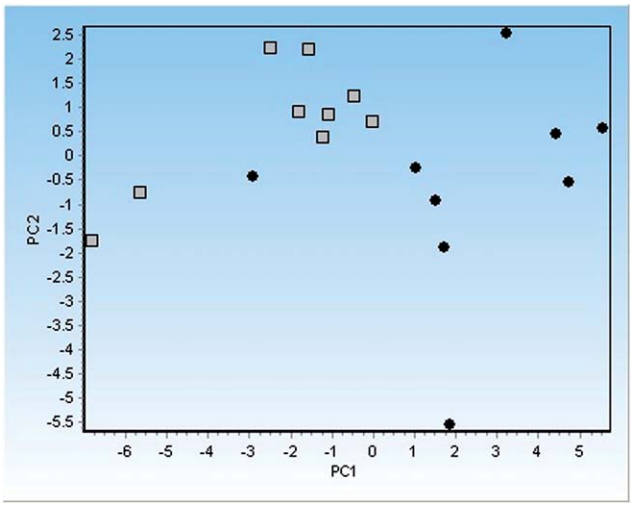

Fig. 3 PCA of 8 regulated miRNAs from single assay PCR plus 15 regulated target genes from mRNA expression analysis.

but miRNAs are known to be highly homologous between different species. These human PCR panels were shown to be a valid and cost-effective method compared to other screening strategies like Next Generation Sequencing (NGS) techniques or microarrays. Herein, the total experimental cohort should be investigated on the array to gain valid results for physiological discussions. When measuring only random samples, the expression of prominent miRNA candidates should be validated by single assay qPCR using the whole experimental cohort.

miRNAs are known to exhibit regulatory effects on a variety of physiological processes in the liver like proliferation and nutrient metabolism. These pathways are also known to be influenced by anabolic treatment[34] and miRNA dysregulation might be a key factor.[7] Several studies showed aberrant expression of different miRNAs like miR-34a, miR-15a and miR-29c, in HCC, in comparison to normal cells. miR-34a represses the translation of anti-apoptotic factors like bcl-2 and therefore stimulates apoptosis.[28] The down-regulation of miR-34a observed in the actual study could indicate an inhibition of apoptosis and could account for the proliferative response following anabolic treatment. Another factor associated with proliferation is miR-15a, which is a known suppressor of uncontrolled cell growth. Its dysregulation has been correlated with different pathophysiological events in the liver, which are associated with uncontrolled proliferation and which are also known side-effects of anabolic misuse like HCC or polycystic liver disease.[7] The down-regulation detected in the actual work could indicate unarrested cell cycle. Also, miR-181c, which has been implicated in uncontrolled proliferation[29] has been demonstrated to be significantly down-regulated. There are no data for miR-181c considering tumor development in liver, but its down-regulation has been detected in different kinds of gastric cancer. Herein, miR-181c has been shown to target different oncogenic factors and the missing repression would therefore lead to gastric carcinogenesis.[30] miR-181c might play a similar role in liver possibly leading to uncontrolled proliferation. Additionally, miR-103 and miR-106a, which have both been found to be up-regulated, have been defined as oncogenic miRNAs inducing proliferation.[7,19] miR-320, which showed a trend for down-regulation, is implicated in the regulation of IGF-1 mRNA expression by decreasing mRNA stability and inducing translational repression.[17] In the mRNA expression analysis of this study, an up-regulation of IGF-1 could be found.[34] These results indicate that the amount of IGF-1 mRNA is increased and additionally the half-life of this mRNA is expanded due to the down-regulation of miR-320. Therefore, the strong proliferative IGF-1 response due to the application of anabolic steroids seems to be mediated both on the transcriptional and the post-transcriptional level. Taken all results of proliferative and tumorigenic factors together, the observed changes in miRNA expression indicate a trend *versus* proliferation in the liver with a risk for tumorigenesis due to uncontrolled cell turnover after the application of anabolic steroid hormones.[31–33] The same conclusions have already been drawn from regulations on the mRNA level.[34]

The trend for up-regulation observed for miR-29c could possibly display one function of the miR-29 family. In diabetes research, the miR-29 family has been implicated in the repression of insulin-stimulated glucose uptake in storage organs. mRNA expression analysis suggests a lower insulin responsiveness of the liver due to the down-regulation of both insulin receptors (IRα and IRβ).[34] These observations on mRNA and miRNA level indicate a lower glucose uptake into the liver. Thus, glucose would remain in the blood flow and could be used as an energy substrate in other target organs *e.g.* muscle cells for proliferative processes due to anabolic treatment.

For pattern recognition of the gained gene expression data, PCA has been applied as a multivariate analysis method, which can be used, if more than three components should be taken into account. In addition to discussed regulations of single genes, this illustration could help us to reveal the transcriptional shift in the treatment group, which could be an indication for functional changes in the liver physiology following anabolic treatment. PCA has already been successfully applied for biomarker research in the detection of anabolic misuse.[13,22,34] The best separation between the control and the treatment group could be reached by combining regulated target genes of mRNA and miRNA of the same transcriptome. It can be observed that both groups arrange together and that a difference between control and treatment group can be monitored suggesting that this approach is promising for biomarker research in the surveillance of anabolic misuse.

Conclusion

For the first time, the miRNA expression profile has been examined under the influence of anabolic steroids in bovine species. Herein, a trend towards proliferation and cell growth as well as a lower insulin responsivity of the liver could be demonstrated on the basis of transcriptional changes. These results from miRNA expression analysis supported the physiological discussions drawn for mRNA from the same transcriptome[34] showing distinctly that also changes in the miRNA expression could be a good indicator for anabolic treatment.

In this study, PCA has been the method of choice for pattern recognition in gene expression results. Control animals could be distinctly separated from treated animals when plotting results from mRNA and miRNA from the same transcriptome indicating that the combination of gene expression results from

mRNA and miRNA seems to be a promising approach to find a gene expression pattern, which might be used for anabolic treatment screening.

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