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Development of mRNA patterns for screening of anabolic steroids in bovine and primate tissues

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Abbreviations

aCP1	acid phosphatase 1
ACTA2	actinα 1
ACTB	actinβ
ADRBK2	adrenergic β kinase 2
AR	androgen receptor
BCL-2	B-cell CLL/lymphoma 2
BCL-XL	B-cell lymphoma extra large
bp	base pairs
C	control
Casp	caspase
CC	carrier control
cDNA	complementary DNA
CK	creatine kinase
CK8, 18	cytokeratin kinase 8, 18
CP2	transcription factor CP2
Ct	threshold cycle
DEGMBE	diethylenglycolmonobutylether
DMSO	dimethylsulfoxid
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleosidtriphosphate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EITR	estrogen induced transcription factor
ER	estrogen receptor
Fas	TNF receptor superfamily member 6
FasL	TNF receptor superfamily member 6 ligand
FGF	fibrobast growth factor
FGFBP	fibrobast growth factor binding protein
for	forward
GAPDH	glycerinealdehyde-3-phosphate dehydrogenase
GR	glucocorticoid receptor
HRE	hormone responsive element
IFN	interferone
IGF-1	insulin like growth factor 1
IGF-1R	insulin like growth factor 1 receptor
IGFBP3	insulin like growth factor binding protein 3
IL	interleukin
LAP	lingual antimicrobial peptide
LTF	lactoferrin
MGA	melengestrolacetate
MHC	major histocompatibility complex
mRNA	messenger RNA
MTPN	myotropin
NTC	no template control
OD	optical density
PCA	principle components analysis
PCR	polymerase chain reaction
pmol	picomol
PR	progesterone receptor
RT-qPCR	quantitative reverse transcription-polymerase chain reaction
RBM	RNA binding protein
rev	reverse
RG	reference gene
RIN	RNA integrity number
RNA	ribonucleic acid
RSA	Republic of South Africa
RT	reverse transcription
SARM	selective androgen receptor modulator
SD	standard deviation
SERM	selective estrogen receptor modulator

T1	one time treated group
T3	three times treated group
TBA	trenbolone acetate
Testo	testosterone
TGF	tumor growth factor
TMOD	tropomodulin
TNF	tumor necrose factor
TNFR	tumor necrose factor receptor
UB3	ubiquitin 3
USF	upstream transcription factor
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide

Zusammenfassung

Natürliche Steroidhormone werden ausgehend von Cholesterin gebildet und sind in die endo- und parakrine Wachstumsregulation verschiedener Gewebe involviert. Einzelne Steroidhormone, wie Östrogene und Androgene wirken anabol, indem sie die Proteinretention im Körper verbessern und Fettreserven abbauen, was zu einer Erhöhung der Wachstumsrate führt. In der Tiermast werden diese anabolen Eigenschaften genutzt, um die Gewichtszunahme und die Futterverwertung zu verbessern, womit die Produktivität erhöht und Kosten gesenkt werden.

In einigen Ländern, wie den USA, Kanada, Australien, Mexiko und Südafrika ist der Gebrauch von Wachstumsförderern in der Tiermast zugelassen. Aufgrund erwiesener Nebenwirkungen für den Konsumenten ist der Gebrauch anaboler Substanzen in der EU verboten, wo die Einhaltung dieser Richtlinie (88/146/EEC) streng überwacht wird.

Ein weiteres Anwendungsgebiet anaboler Steroidhormone ist die Behandlung altersbedingter Krankheiten, wie Osteoporose oder Sarkopenie, welche durch eine Abnahme der endogenen Produktion von Östradiol und Testosteron bei rückläufiger Gonadenfunktion verursacht werden. Für die Behandlung dieser altersbedingten Krankheiten wurden so genannte selektive Androgen Rezeptor Modulatoren (SARM) entwickelt. Darunter versteht man synthetische Moleküle, welche die nützlichen zentralen und peripheren Eigenschaften von Testosteron besitzen, jedoch kaum Nebenwirkungen aufweisen. Aufgrund der positiven Wirkungen eines SARM auf die Muskelmasse ist das Risiko des Missbrauchs dieser Substanzen in der Tiermast oder im Sport vorhanden.

Um den Missbrauch anaboler Substanzen in der Tiermast oder im Sport zu kontrollieren, werden Hormonrückstände mittels Immunoassays oder chromatographischer Methoden in Kombination mit Massenspektrometrie detektiert. Mit Hilfe dieser Methoden können nur bekannte Substanzen nachgewiesen werden. Um eine effiziente Kontrolle des Missbrauchs anaboler Stoffe zu gewährleisten, ist es nötig neue Technologien zu entwickeln, mit welchen man den Gebrauch einer breiten Masse an illegalen Medikamenten, inklusive neu entwickelter Xenobiotika nachweisen kann.

Ein Ansatz zur Entwicklung einer neuen Nachweismethode ist das Aufzeigen physiologischer Effekte, welche durch die Einnahme anaboler Substanzen verursacht werden. Ein viel versprechender Weg solche physiologischen Veränderungen nachzuweisen, ist die Bestimmung von Veränderungen in der mRNA Expression mittels quantitativer real-time RT-PCR (RT-qPCR).

Ziel dieser Arbeit war es zu prüfen, ob die Bestimmung von Genexpressionsveränderungen Potential für die Entwicklung neuer Technologien zum Nachweis missbräuchlicher Anwendung anaboler Substanzen hat. Hierfür wurde die

mRNA Expression steroidabhängiger Gene im Blut und in vaginalen Epithelzellen – Gewebe, welche leicht vom lebenden Individuum genommen werden können - mittels RT-qPCR quantifiziert und mögliche Veränderungen statistisch bewertet.

In allen drei Tierversuchen, die im Rahmen dieser Arbeit durchgeführt wurden, konnten Genexpressionsveränderungen festgestellt werden. In zwei dieser Studien konnte aus den signifikant regulierten Genen mit Hilfe biostatistischer Methoden, wie der Principle Components Analyse (PCA) oder der hierarchischen Clusteranalyse eine Trennung von Kontrollgruppe und Behandlungsgruppe dargestellt werden.

Die Ergebnisse dieser Arbeit zeigen, dass die Quantifizierung von Genexpressionsveränderungen eine vielversprechende Herangehensweise für die Entwicklung neuer Technologien zum Nachweis des missbräuchlichen Gebrauchs anaboler Substanzen darstellt.

Abstract

Natural steroid hormones are synthesized from cholesterol and are involved in endocrine and paracrine regulation of growth in different tissues. Some steroid hormones like androgens and estrogens have anabolic functions by enhancing body protein accretion and mobilizing fat stores, which results in an increased growth rate. These properties are useful in animal husbandry to improve weight gain and feed efficiency and thereby increase productivity and reduce costs. In some countries like the USA, Canada, Australia, Mexico and South Africa the use of growth promoters is approved. Due to proven negative side effects for consumers the use of anabolic substances is forbidden in the EU, where the compliance of this directive (88/146/EEC) is strictly controlled.

Another application area of anabolic steroid hormones is the treatment of age related diseases like osteoporosis or sarcopenia, which are related to a decrease of the endogenous production of anabolic steroid hormones during diminishing gonade function, mainly estradiol and testosterone. For the treatment of these age related diseases, synthetic molecules called selective androgen receptor modulators (SARMs) are developed, which have the potential to mimic the desirable central and peripheral androgenic anabolic effects of testosterone but with less side effects. Due to the positive effects on muscle strength of SARMs the risk of the misuse of these substances in animal husbandry or human sports as anabolic agent is present.

To uncover the abuse of anabolic agents in animal husbandry or human sports, hormone residues are detected by immuno assays or chromatographical methods in combination with mass spectrometry. With these methods only known substances can be discovered. To enable an efficient tracing of unknown 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.

Verifying physiological effects caused by anabolic agents will be a new way to develop potential monitoring systems. The determination of changes in mRNA expression by quantitative real-time RT-PCR (RT-qPCR) is a promising approach to verify those physiological changes.

The aim of this thesis was to proof the potential of the determination of mRNA expression changes for the development of a screening method to detect the misuse of anabolic steroid hormones. Therefore expression changes of steroid responsive genes that were selected by screening the actual literature were quantified in blood and vaginal epithelial cells – tissues that can easily been taken from the living individual. Gene expression changes were measured by RT-qPCR.

In all three animal trials included in this thesis, expression changes of multiple genes in blood and bovine vaginal smear could be quantified. In two studies, biostatistical tools, like Principle Components Analysis (PCA) or Hierarchical Cluster Analysis were successfully used to distinguish treated and untreated animals by involving all significantly regulated genes.

The results of this thesis indicate that the quantification of gene expression changes is a promising approach for the development of new screening methods to trace the abuse of anabolic agents.

1 Introduction

1.1 Anabolic Steroid Hormones – use and misuse in animal husbandry

Natural steroid hormones are synthesized from cholesterol and can be classified in five subgroups: mineralocorticoids, glucocorticoids, gestagens, androgens, and estrogens and are involved in endocrine and paracrine regulation of different tissues. Some steroid hormones like androgens and estrogens have anabolic functions by enhancing body protein accretion and mobilizing fat stores, which results in an increased growth rate [1, 2]. 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 behaviours like courtship and improves growth of skeletal muscle which is important for defending the territory [3, 4].

In animal husbandry the myotropic, growth promoting properties of steroid hormones are beneficial. Used orally, the natural steroid hormones testosterone and estradiol are almost inactive. Besides these natural steroids also 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. As only MGA is orally active, the other drugs have to be applied by implantation [5]. In meat production growth promoters are used to increase productivity and to reduce costs by improving weight gain and feed efficiency [6, 7]. The use of growth promoters is approved in some countries like the USA, Canada, Australia, Mexico, and South Africa. It has been proven that hormone residues in meat are increased and have adverse side effects for the consumer [8-11]. 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. To enforce the directive (88/146/EEC), permanent surveillance is essential [12-17].

1.2 Steroid Hormones in Hormone Replacement Therapy

Over the last decades the proportion of elderly people in the population has increased [18]. This is the reason why the incidence of age related conditions like sarcopenia (loss of muscle mass) and osteoporosis (loss of bone density) is rising and becoming one of the major topics in health care [19-22]. The combination of sarcopenia and osteoporosis

results in a high incidence of bone fractures relating to accidental falls, which is a significant cause of morbidity in the elderly population. Both conditions are related to the decrease in the endogenous production of anabolic steroid hormones, mainly estradiol and testosterone [23]. Hormone replacement therapy is a major topic in the treatment of frailty. Men and women suffering from frailty are treated with testosterone or estradiol but both therapies are associated with various side effects, like skin virilization in women, prostate hypertrophy in men and an increased risk of cancer [24-26]. 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 [27]. An “ideal” SARM or SERM is an orally active compound that provides an increase in muscle mass and strength and has a positive effect on bone density without inducing undesirable side effects [28]. Due to the positive effects on muscle strength of SARMs and SERMs the risk of the misuse of these substances as anabolic agent is present.

1.3 Potential of transcriptomics for biomarker development to trace anabolic steroid hormone functions

To uncover the abuse of anabolic agents in animal husbandry hormone residues are detected using immuno assays or chromatographical methods in combination with mass spectrometry [29-32]. 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.

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 [33, 34]. To develop such biomarkers *omic* technologies, like transcriptomics, proteomics and metabolomics are applied [35-37].

The use of these *omic* technologies to develop biomarker patterns by tracing anabolic steroid hormone functions will be a promising way to develop a new screening method for the detection of the misuse of anabolic agents [38].

Steroid hormone receptors belong to the family of nuclear receptors and show a high affinity to their corresponding hormone [39, 40]. 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 [41]. 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 [42-44]. Binding of the ligand results in a conformational change leading 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) [40, 45, 46]. After DNA binding, different coregulators that are recruited to activate transcription of target genes. Figure 1 shows the main steps in steroid hormone action.

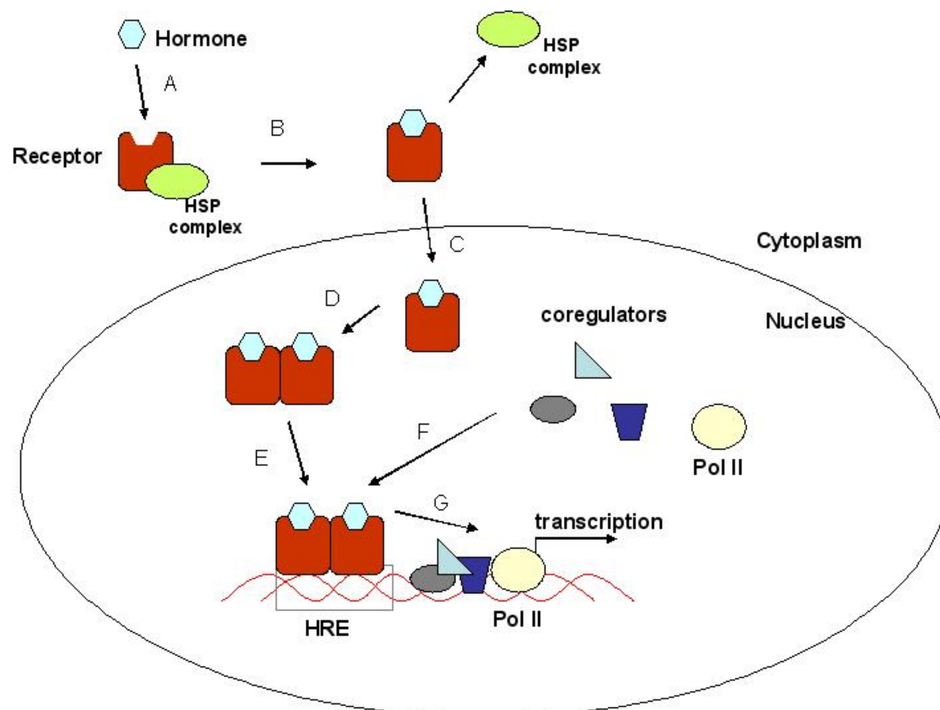

Figure 1: Schematic diagram of the activation of a cytoplasmic steroid hormone receptor
After hormone binding (A) the HSP complex dissociates from the receptor (B), the hormone receptor complex translocates to the nucleus (C), dimerizes (D) and binds to a hormone responsive element (HRE) in the promoter region of a specific gene (E). After binding to the HRE different coregulators of transcription are recruited (F), which are responsible for transcriptional activation [47, 48].

Steroid hormones not solely regulate gene transcription activity, but also influence the stability of generated mRNA. They are able to stabilize or destabilize specific mRNAs. 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 [49].

1.4 Aims

The objective of this thesis was to proof the potential of transcriptomics technology for the development of a screening method to detect the misuse of anabolic steroid hormones. Therefore three different animal trials were employed. Two studies on female cattle, where the effects of different combinations of steroid hormones on gene expression in blood and vaginal smear was quantified and one study on cynomolgus monkeys (*Macaca fascicularis)* where the effects a novel SARM on gene expression of whole blood was compared to the effects of natural testosterone. In all three animal trials expression changes of steroid responsive genes that were selected by screening the actual literature were quantified. Gene expression changes were measured by RT-qPCR. Biostatistical tools, like PCA or Hierarchical Cluster Analysis could be helpful to proof, if quantified gene expression changes will be promising biomarkers for the development of a new screening system to detect the misuse of anabolic agents. Figure 2 presents a schematic overview of the transcriptomic approach to trace anabolic hormone functions.

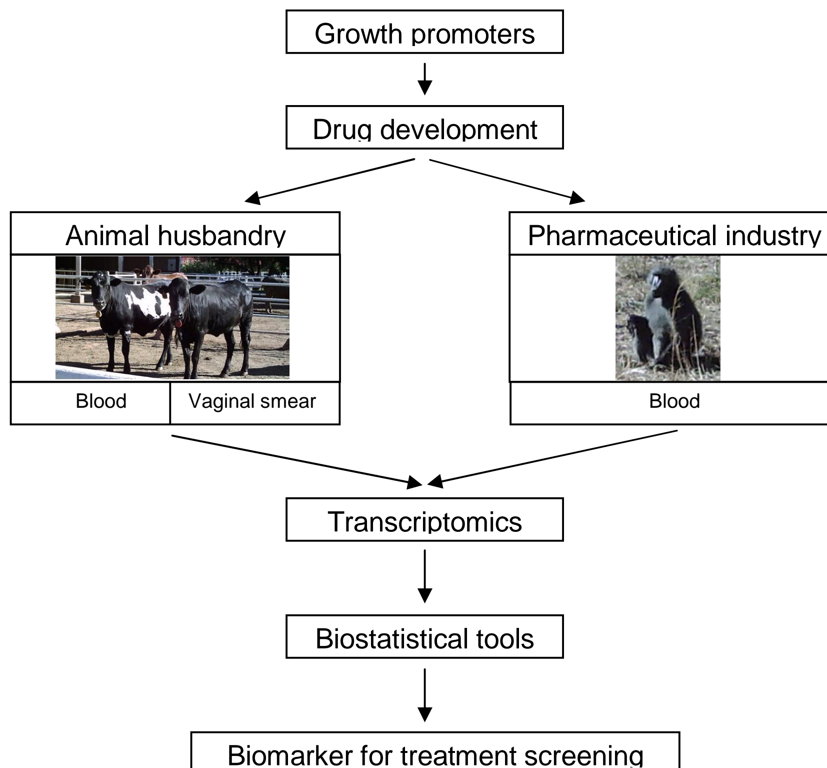

Figure 2: Scheme of the use of transcriptomics to trace anabolic hormone functions.

2 Materials and Methods

2.1 Animal Experiments

Anabolics study on Nguni Cattle

To test the potential to use transcriptomics for the development of a new sensitive test system to screen for the misuse of anabolic agents in food producing animals, an animal study that represents the situation in praxis had to be organized. In the Republic South Africa the use of certain anabolic agents for improvement of growth of meat producing animals is permitted. Anabolic preparations are available on the market there. Therefore it was easier to get the permission for such an animal study in South Africa than it would be in Germany. So it was part of this PhD thesis to organize all steps (study design, sampling of blood and vaginal smear, tissue sampling at slaughter) of an animal study in cooperation with the Onderstepoort Veterinary Institute in Pretoria, South Africa. A journey to Pretoria of two weeks for organizing the transfer of the samples to Germany and for organizing and performing tissue sampling at slaughter was part of this PhD thesis.

Earlier animal studies that were performed to analyze the effects of anabolic substances on growth performance in cattle showed, that 5-10 animals per group are adequate if the natural variance between the animals is minimized by using animals within one breed, one gender and of similar age [50]. It is assumed that gene expression will vary like growth performance and so this aspect was considered in the planning of the study.

Regarding the present conditions in Onderstepoort it was possible to include 18 animals in the trial. The healthy, non pregnant, two year old Nguni heifers were separated into two groups of nine animals each (n = 9). One group was treated with Revalor H® (140 mg Trenbolone acetate plus 14 mg estradiol; Intervet, Isando, RSA) by implantation into the middle third of the pinna of the ear and one group was untreated and served as control.

For this PhD Thesis, whole blood and vaginal smear samples were taken at four time points. Predose samples were taken after study start without prior treatment. Further samples were taken at day 2, day 16 and day 39 of treatment. At the same time points a complete blood count was done by the section of clinical pathology, University of Pretoria, South Africa, to control the health status of the animals. Blood samples for gene expression analysis were taken as described previously [51]. Vaginal smear was taken using a sterilized spoon. The smear was directly transferred into TriFast (Peqlab, Erlangen, Germany) and stored at -80°C. The animal attendance and blood sampling were done by the Onderstepoort Veterinary Institute (Onderstepoort, Pretoria, South Africa). The animals were housed and fed according to good veterinary practice.

Pour on anabolics study on veal calves

To test the potential of the identified biomarker candidates from the anabolics study on Nguni cattle a second study in which animals are treated with an illicit hormone cocktail was conducted. The application of such hormone cocktail is not allowed, even in countries where the use of anabolic agents in food producing animals is permitted, and so the performance of this trial required a special permission from the government. This trial was organized in cooperation with the RIKILT Institute of food safety, Wageningen, Netherlands. The design of the study in cooperation with the RIKILT Institute of food safety, the study performance in Freising-Weihenstephan and sampling of blood, hair, urine and different tissues at slaughter was part of this PhD thesis.

In planning such animal experiments there are many factors that have to be considered especially concerning the number of animals included in the trial. The guidelines for that trial was to have four different groups. Regarding the present conditions at the Versuchsstation Veitshof, Freising, Germany it was possible to include 20 animals in the trial. The trial was permitted by the Regierung von Oberbayern (Reference Number 55.2-1-5412531.8-102-07). A statistical report required for the application for that animal trial demonstrates that by including two control groups and two groups treated with different doses of hormones, 5 animals per group are adequate for statistical analysis.

So, 20 healthy, 5-7 weeks old Holstein Friesian calves were separated into four groups of five animals each (n=5).

The hormone mix for this pour on study contained 25 mg Oestradiolbenzoate (Intervet, Boxmeer, Netherlands), 60 mg Testosteronedecanoate (Organon, Oss, Netherlands) and 60 mg Testosteronecypionate (Organon). The hormone mix was applicated in two different ways: via injection (one animal per group) or via pour on (four animals per group). To ensure the transit of the hormone mix through the skin, following carriers were used: Ivomec (Merial BV, Amstelveen, Netherlands), Dimethylsulfoxid (DMSO) (Sigma-Aldrich, Zwijndrecht, Netherlands), Miglyol 840 (Dynamit Nobel, Germany) und Diethylenglycolmonobutylether (DEGMBE) (Merck, Amsterdam, Netherlands). For injection arachide oil served as carrier.

The first group served as untreated control group (C). In the second group the animals were treated only with the different carriers three times in weekly application (CC). In the third group the animals were treated once with the different carrier hormone mixes (T1). The animals of the fourth group were treated with the different carrier hormone mixes three times in weekly application (T3). To treat the animals via pour on, they were shaven on the back from neck to tail. For treatment 10 mL of hormone carrier mix were administered at the shaved region. The injected animals were treated intramuscular in the neck. Table 1 shows a scheme of the different treatment groups.

Table 1: Treatment scheme of the pour on animal trial

Group	Animal	Treatment	Group	Animal	Treatment
C	1	none	T1	11	Injection hormones
	2	none		12	DEGMBE+hormones
	3	none		13	DMSO+hormones
	4	none		14	IVOMEC+hormones
	5	none		15	Miglyol+hormones
CC	6	Injection Arachide oil	T3	16	Injection hormones
	7	DEGMBE		17	DEGMBE+hormones
	8	DMSO		18	DMSO+hormones
	9	IVOMEC		19	IVOMEC+hormones
	10	Miglyol		20	Miglyol+hormones

Blood samples were taken at eight different time points: Predose samples were taken after study start without prior treatment. Further samples were taken at day 2, day 7, day 14, day 21, day 35, day 63, and day 91 of treatment. Blood samples were taken as described previously [52].

During the treatment period the animals were weighted 4 times (before treatment and at days 28, 63, and 91 of treatment).

The animal attendance and blood sampling were done at the Veitshof, Institute of Physiology, Technical University of Munich, Freising-Weihenstephan, Germany. The animals were housed and fed according to practice.

SARM study on Macaca fascicularis

The aim of this study was to test the possibility of finding gene expression biomarkers for the treatment with a new SARM. Therefore samples for gene expression analyses were taken from an animal trial that was organized within the second phase clinical trial by TAP Pharmaceuticals, Chicago, USA.

In that trial 24 male cynomolgus monkeys (*Macaca fascicularis*) were separated into four groups of six animals each. All animals were 5-6 years old, skeletally mature and had an average body weight of 6 ± kg. The treatments were control: oral vehicle, Testo: 3.0 mg/kg Testosteronenanthate as Testoviron®-depot-250 (Schering, Berlin, Germany) dosed biweekly by intramuscular injection, SARM1: 1 mg/kg SARM LGD2941 daily, oral and SARM10: 10 mg/kg SARM LGD2941 daily, oral. The oral vehicle control and the SARM were dosed once daily for 90 days.

Whole blood samples were taken at three time points. Predose samples were taken after study start without prior treatment. Further samples were taken at day 16 and day 90 of treatment. Duplicate blood samples were taken as described previously [53].

The animal attendance and blood sampling were done by Covance Laboratories GmbH (Münster, Germany) and was conducted with permission from the local veterinary authorities and in accordance with accepted standards of Human Animal Care.

2.2 RNA Extraction and Quality Determination

RNA from blood samples was extracted using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany). This system is designed for the stabilization and extraction of RNA from human whole blood. Before using it for *Macaca fascicularis* or cattle it had to be tested, if this system also works for these species. Due to the high homology between human and primate blood the PAXgene system worked well for *Macaca fascicularis.* The cellular composition of bovine blood differs from that of human blood. There are for example no reticulocytes present, because the maturation of bovine erythrocytes completely takes place in the bone marrow. For testing the PAXgene system with bovine blood, several blood volumes were used. The RNA yield was generally lower compared to the RNA extracted from human blood. It was not practicable to use more than 2.5 ml bovine blood, because by using higher volumes, the stabilizer was not able to lyse all cells and so erythrocytes accumulated and RNA extraction was not possible. Therefore RNA extraction from bovine blood was performed according to the manufacturer´s instructions.
There is no information available about the extraction of RNA from bovine vaginal epithelial cells. The literature only describes how to extract RNA from vaginal epithelial cells obtained from euthanized mice. Our intention was to extract RNA from vaginal epithelial cells of living cattle. A method to get vaginal epithelial cells is to take vaginal smear containing keratinized epithelial cells. The high amount of cervical mucus present in bovine vagina at the end of the estrous cycle was problematic for sampling but by using a sterilized spoon sampling could be successfully performed.
For RNA extraction several systems were tested including phenol based methods and kits using silica membranes. After comparing RNA yield and RNA quality, the method of choice was peqGold Tri-Fast (PeqLab Biotechologies) based on the manufacturer´s instructions.
To quantify the amount of total RNA extracted, optical density (OD) was measured with the Biophotometer (Eppendorf, Hamburg, Germany) or with the NanoDrop 1000 (PeqLab Biotechnologies) for each sample. RNA purity was calculated with the $OD_{260/280}$ ratio.
RNA integrity and quality control was performed via automated capillary electrophoresis in the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). Eukaryotic total RNA Nano Assay (Agilent Technologies) was taken for sample analysis and the RNA Integrity

Number (RIN) served as RNA quality parameter. Agilent 2100 Bioanalyzer calculated the RIN value based on a numbering system from 1 to 10 (1 being the most degraded profile, 10 being the most intact) for all samples.

2.3 Selection of Target Genes

Candidate genes that might be biomarkers in blood or vaginal smear were chosen by screening the respective literature for steroid related effects on blood or vaginal epithelial cells. Quantified target genes are listed in tables 2-4.

Table 2: Description and accession number of target genes that were quantified in bovine blood [54-58]

Gene Group	Target Genes	Accession Number
Reference Genes	Histone H3	NM_001014389
	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ)	NM_174814
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	U85042
	Ubiquitin 3 (UB3)	Z18245
Steroid receptors	Androgen receptor (AR)	AY862875
	Estrogen receptors α and β (ERα and Erβ)	NM_001001443 NM_174051
	Glucocorticoid receptor α (GRα)	AY238475
Apoptosis regulators	TNF receptor superfamily member 6 (Fas)	U34794
	TNF receptor superfamily member 6 ligand (FasL)	XM_584322
	B-cell CLL/lymphoma 2 (BCL-2)	XM_586976
	B-cell lymphoma-extra large (Bcl-Xl)	AF245489
	Tumor necrosis factor α (TNFα)	NM173966
	Tumor necrosis factor receptor 1 and 2 (TNFR1, TNFR2)	NM173966 AF031589
	Caspase 3 and 8 (Casp3, Casp8)	NM_001077840 DQ319070
Interleukins	Interleukins 1α, 1β, 6, 8, 10, 12B (p40) and 15 (IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12B, IL-15)	M36182. M37211 NM173923 AF232704 NM_174088 NM_174356 NM_174090
CD Antigens	CD 4, 8 and 14	NM_001103225 BC151259 NM_174008
Growth factors	Insulin-like growth factor 1 (IGF-1)	NM_001077828
	Tumor growth factor β (TGFβ)	XM592497
	Interferon gamma (IFN-γ)	NM_174086
others	Inflammatory factor nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFkB)	NM_001076409
	Actinβ (ACTB)	AY141970
	Actin α 1 (ACTA1)	NP_776650
	Creatine Kinase (CK)	NM_174225
	Adrenergic beta kinase 2 (ADRBK2)	NM_174500
	Major histocompatibility complex class II (MHC II)	NM_001034668
	Jun oncogene(JUN)	NM_001077827
	Estrogen induced transcription factor (EITr)	XR_027981
	Myotrophin (MTPN)	NM_203362
	Tropomodulin 3 (TMOD3)	NM_001075987
	RNA binding protein 5 (RBM5)	NM_001046374

Table 3: Description and accession number of target genes that were quantified in bovine vaginal smear [59-66]

Gene group	Target Genes	Accession Number
Reference Genes	Histone H3	NM_001014389
	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ)	NM_174814
Steroid Receptors	Androgen receptor	AY862875
	Estrogen receptor α (ERα)	NM_001001443
	Progesteron receptor (PR)	XM_583951.4
Keratinization Factors	Fibroblast growth factor 7 (FGF7)	XM_869016
	Fibroblast growth factor binding protein (FGFBP)	NM_174337.2
	Cytokeratin 8 (CK8)	BC103339
	Cytokeratin 18 (CK18)	XM_582930
Growth Factors	Epithelial growth factor (EGF)	AY195611.1
	Epithelial growth factor receptor(EGFR)	XM_592211.4
	Insulin like growth factor 1 (IGF-1)	NM_001077828
	Insulin like growth factor 1 receptor (IGF-1R)	X54980
	Insulin like growth factor binding protein (IGFBP3)	NM_174556.1
	Tumor growth factor a (TGFα)	XM_593710.4
	Lactoferrin (LTF)	NM_180998.2
Apoptosis	TNF receptor superfamily member 6 (Fas)	U34794
	TNF receptor superfamily member 6 ligand (FasL)	XM_584322
	Caspase 3 and 8 (Casp3, Casp8)	NM_001077840 DQ319070
	Tumor necrosis factor α (TNFα)	NM173966
	Tumor necrosis factor receptor 1 (TNFR1)	NM173966
Interleukins	Interleukins 1α, 1β, (IL-1α, IL-1β)	M36182 M37211
Oncogens	c jun	AF069515
	c fos	AF069515
Others	Ubiquitin 3 (UB3)	Z18245
	Actinβ (ACTB)	AY141970
	Lingual antimicrobial peptide (LAP)	NM_203435

Table 4: Description and accession number of target genes that were quantified in primate blood [55, 56, 67-69]

Gene Group	Target Genes	Accession Number
Reference Genes	Actinβ (ACTB)	NM_001101
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_002046
	Ubiquitin 3 (UB3)	NM_021009
Apoptosis regulators	TNF receptor superfamily member 6 (Fas)	NM_000043
	TNF receptor superfamily member 6 ligand (FasL)	NM_000639
	B-cell CLL/lymphoma 2 (BCL-2)	NM_000633
	B-cell lymphoma-extra large (Bcl-Xl)	NM_138578
	Tumor necrosis factor α (TNFα)	NM_000594
	Tumor necrosis factor receptor 1 and 2 (TNFR1, TNFR2)	NM_001065 NM_001066
	Caspase 3 and 8 (Casp3, Casp8)	NM_004346 NM_001228
	CD 30 ligand (CD30L)	NM_001244
Interleukins	Interleukins 1β, 2, 4, 6, 10, 12B (p40), 13 and 15 (IL-1β,IL-2, IL-4, IL-6, IL-10, IL-12B, IL-13, IL-15)	NM_000576 NM_000586 NM_172348 NM_000600 NM_000572 NM_002187 NM_002188 NM_172174
CD Antigens	CD 4, 8, 11b, 14, 20, 25 and 69	NM_000616 NM_001768 NM_000632 NM_000591 NM_021950 NM_000417 NM_001781
Growth factors	Insulin-like growth factor 1 receptor (IGF-1R)	NM_000875
	Tumor growth factor β (TGFβ)	NM_000660
inflammatory factors	Inflammatory factor nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFκB)	NM_003998
	NFκB inhibitor (IκB)	NM_020529
reticulocyte genes	Haemoglobin alpha (α-globin)	NM_000517
	Haemoglobin beta (β-globin)	NM_000518
	Acid phosphatase 1 (αCP1)	NM_006196
	Upstream transcription factor 1 (USF-1)	NM_007122
	Transcription factor CP2 (CP2)	NM_005653
other genes	Androgen receptor (AR)	NM_000044
	Tumor necrosis factor β (TNFβ)	NM_000595
	CD27 ligand (CD27L)	NM_001252

2.4 Specific Primer Design

All bovine primers were designed using published bovine nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). There are almost no nucleic acid sequences available for *Macaca fascicularis.* For primer design human sequences of the target genes were used. Primer design using human sequences was not possible for all target genes. In this case homologue sequence parts between different species, like mouse, rat and humans were detected using the sequence alignment tool (bl2seq) of the National Center for Biotechnology Information (NCBI) and primer pairs were designed including sequences of the detected homologue parts.
Primer design and optimization was done with primer design program of MWG Biotech (MWG, Ebersberg, Germany) and primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with regard to primer dimer and self-priming formation. Newly designed primers were ordered and synthesized at MWG Biotech (Ebersberg, Germany). Primer testing was performed with three optional samples and a no template control (NTC contains only RNAse free water). To determine the optimal annealing temperature for each primer set a temperature gradient PCR was done.

2.5 Two-Step RT-qPCR Analysis

For the studies on bovine tissues, two step RT-qPCR was performed.

RNA Reverse Transcription

Constant amounts of 500 ng or 1 µg RNA were reverse transcribed respectively to cDNA using the following RT master mix: 12 µL 5×Buffer (Promega, Mannheim, Germany), 3 µL Random Hexamer Primers (50 mM; Invitrogen, Carlsbad, USA), 3 µL dNTP Mix (10 mM; Fermentas, St Leon-Rot, Germany) and 200U of MMLV H- Reverse Transcriptase (Promega) according to the manufacturer's instructions.

qPCR Analysis

To analyze gene expression of candidate genes, RT-qPCR analysis was done with the iQ5 (Bio-Rad, Munich, Germany), using MESA GREEN qPCR MasterMix Plus for SYBR® Assay w/fluorescein Kit (Eurogentec, Cologne, Germany) by a standard protocol, recommended by the manufacture.

With the kit a PCR master mix was prepared as follows: For one sample it is 7.5 µL MESA GREEN 2x PCR Mix, 1.5 µL forward primer (10 pmol/µl), 1.5 µL reverse primer (10 pmol/µl) and 3 µL RNAse free water. For qPCR analysis 1.5 µL cDNA was added to 13.5 µL PCR master mix (total PCR mix: 15µL). qPCR was performed in 96 Well Plates (Eppendorf) and pipetting was done by the epMotion 5075 (Eppendorf).
The following real-time PCR cycling protocol was employed for all investigated factors: denaturation for 5 min at 95°C, 40 cycles of a two segmented amplification and quantification program (denaturation for 3 s at 95°C, annealing for 10 s at primer specific annealing temperature listed in table 1), a melting step by slow heating from 60 to 95°C with a dwell time of 10 s and continous fluorescence measurement. Threshold cycle (Ct) and melting curves were acquired by using the iQ5 Optical System software 2.0 (Bio-Rad). Only genes with clear melting curves were taken for further data analysis. Samples that showed irregular melting peaks were excluded from the quantification procedure.

2.6 One-Step RT-qPCR Analysis

For the SARM Study, one step quantitative real time RT-PCR analysis was used, which was performed using SuperScript III Platinum SYBR Green One-Step qPCR Kit (Invitrogen) by a standard protocol, recommended by the manufacture. With the kit a PCR master mix was prepared as follows: For one sample it is 5 µL 2x SYBR Green Reaction Mix, 0.5 µL forward primer (10 pmol/µL), 0.5 µL reverse primer (10 pmol/µL) and 0.2 µL SYBR Green One-Step Enzyme Mix (Invitrogen). 6.2 µL of the PCR master mix was filled in the special 100 µL tubes and 3.8 µL RNA (concentration 1 ng/µL respectively 10 ng/µL) was added (total PCR mix: 10µL). Tubes were closed, placed into the Rotor-Gene 3000 and Analysis Software v6.0 was started (Corbett Life Science, Sydney, Australia). The following uniform one-step RT-qPCR temperature cycling program was used for all genes: Reverse transcription took place at 55°C for 10 min. After 5 min of denaturation at 95°C, 40 cycles of real-time PCR with 3-segment amplification were performed consisting of 15 s at 95°C for denaturation, 30 s at primer dependent temperature for annealing and 20 s at 68°C for polymerase elongation. The melting step was then performed with slow heating starting at 60°C with a rate of 0.5°C per second up to 95°C with continuous measurement of fluorescence.
Threshold cycle (Ct) and melting curves were acquired by using the *"Comparative quantitation"* and *"Melting curve"* program of the Rotor-Gene 3000 Analysis software v6.0. Only genes with clear melting curves were taken for further data analysis. Samples that showed irregular melting peaks were excluded from the quantification procedure.

2.7 Data Analysis and Statistics

Haemogram in bovine studies

Significant changes of the amount of the different blood cells between the treatment groups were determined using an unpaired t-test. Results with $p < 0.05$ were considered as statistically significant.

Weight gain in bovine studies

For the pour on study, significant changes of carcass weight and the weight gain of the different weighting time points relatively to the beginning of the trial was done by comparing the treatment groups to the control group using an unpaired t-test. Results with $p < 0.05$ were considered as statistically significant.

Statistical analysis of gene expression data

Statistical description of the expression data as well as statistical tests were produced with Sigma Stat for the bovine studies and with SAS v. 9.1.3 for Windows (SAS Institute, Cary, USA) for the SARM study. Since the amplification efficiency was not known, the assumption of identical amplification efficiency 100% was made, allowing more simple quantification model [70].

The Ct values of each gene were averaged by arithmetic mean for each animal. The obtained mean Ct values were then translated to normalized expression quantities using two reference genes (RG) in form of normalization index. The normalization index was calculated as an arithmetic mean of the Ct values of the two RG:

$$\text{normalization index} = \text{mean}\,(Ct_{RG1}, Ct_{RG2}) \qquad (1)$$

Then, the expression of every target gene was calculated relatively to the expression of the RG as:

$$\text{normalized expression} = 2^{\text{reference index}} / 2^{\text{Ct target gene}}, \qquad (2)$$

where the 2 represents the 100% amplification efficiency.

For quantification of gene expression in blood samples the normalized expressions of the treatment timepoints were divided with the normalized expressions of the baseline (predose), generating the expression ratio R as:

$$R_{\text{timepoint/baseline}} = \text{normalized expression}_{\text{timepoint}} / \text{normalized expression}_{\text{baseline}} \qquad (3)$$

The expression ratio R for blood and the normalized expression for vaginal smear was then analysed statistically using the t-test. Results with $p < 0.05$ were considered as statistically significant.

Principal components analysis (PCA)

To disclose multivariate response to the treatment, the method of principal components analysis (PCA) was employed using GenEx v. 4.3.x (MultiD Analyses AB, Gothenburg, Sweden). 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. Normalized expression values of all responding genes were taken as the initial variables and reduced to two principal components only, facilitating thus resolution of treatment clusters in the scatter plot.

Hierarchical Cluster Analysis

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 [71, 72]. Hierarchical clustering was employed using GenEx v. 4.3.6 (MultiD Analyses AB).

3 Results and Discussion

3.1 Anabolics study on Nguni Cattle

Haemogram

The haemograms indicate that the animals were healthy. The white blood cell count and the amount of lymphocytes, monocytes, eosinophil, and basophil granolucytes ranged in physiological levels with no significant differences between treatment group and control (p-values are listed in table 5). Therefore significant changes in mRNA expression in blood can be interpreted as real changes in gene expression and are not due to changes in the blood cell, especially the mRNA expressing white blood cells.

Table 5: List of p-values for the regulation of the amount of the different blood cells

Timepoint	white blood cell count	lymphocytes	monocytes	eosinophils	basophils
Predose	0.5347	0.9263	0.1273	0.1914	0.1691
Day 2	0.2827	0.8051	0.8979	0.3663	-
Day 16	0.9310	0.7601	0.0848	0.3551	0.3927
Day 39	0.3758	0.5106	0.4026	0.0690	0.8353

RNA integrity

Good RNA quality is important for the overall success of RNA based analysis methods like real time RT-qPCR [73-76]. The RNA degradation level was determined using the lab-on-a-chip technology of the Agilent Bioanalyzer (Agilent Technologies).

The mean (±SD) RIN value of the blood samples was 8.3 ± 0.3 indicating fully integer total RNA.

The RIN value of the vaginal smear samples was only 4.5 ± 2.02 (mean ± SD). The relatively low RNA quality could be due to the fact that cells found in the vaginal smear are detached, keratinized and partly degraded. Another reason for the low RNA quality results can be RNases present in the vaginal flora. Due to the low RNA quality, the validation of qPCR assays was problematic. Primer pairs had to be designed resulting in PCR products with a length of about 100 bp. This is recommended by Fleige et al for RNA with low quality [77, 78]. Following these guidelines RT-qPCR for 29 genes (27 candidate genes and 2 reference genes) could be successfully established.

RT-qPCR results and data analysis

Blood

Significant regulation of gene expression of the treatment group compared to the control group could be identified for IL-6, MHC II, CK, MTPN and RBM5 after 2 days (figure 3), for GRα, ERα, Fas and IL-1α after 16 days (figure 4) and for ACTB, GRα, IL-1α and IL-1β after 39 days of treatment (figure 5). The resulting p-values and the regulation ratio between control and treatment group are listed in table 6.

Table 6: Significant mRNA expression changes. P-values and x-fold regulation between steroid treatment and control group. Green background indicates down-regulation and red background indicates up-regulation of the genes.

Gene Group	Gene	Timepoint	p-value	x-fold regulation
Steroid receptors	GRα	Day 16	0.0159	1.597
		Day 39	0.0273	1.345
	ERα	Day 16	0.0106	1.509
Apoptosis regulators	Fas	Day 16	0.0463	1.978
Interleukins	IL-1α	Day 16	0.0108	2.268
		Day 39	0.0364	1.65
	IL-1β	Day 39	0.0412	1.475
	IL-6	Day 2	0.0125	0.434
Others	MHCII	Day 2	0.0219	0.682
	CK	Day 2	0.0046	0.637
	MTPN	Day 2	0.0129	0.621
	RBM5	Day 2	0.0353	0.637
	ACTB	Day 39	0.0095	1.345

In the box-whisker plots, differences in gene expression of the control group compared to baseline can be observed. This reflects the natural variability of the non-induced expression in each studied subject.

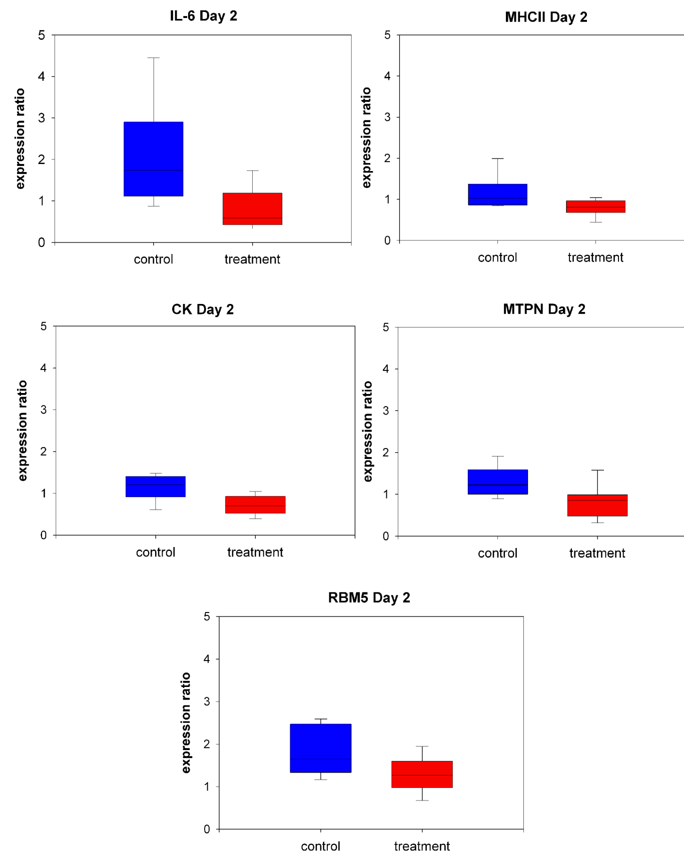

Figure 3: Significant regulations for IL-6 (A), MHCII (B), CK (C), MTPN (D), and RBM5 (E) between control and treated samples after 2 days of treatment.

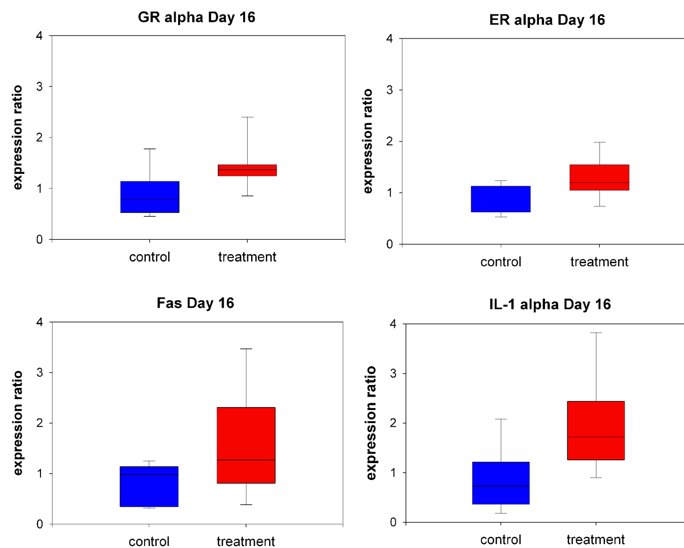

Figure 4: Significant regulations for GRα (A), ERα (B), Fas (C) and IL-1α (D) between control and treated samples after 16 days of treatment.

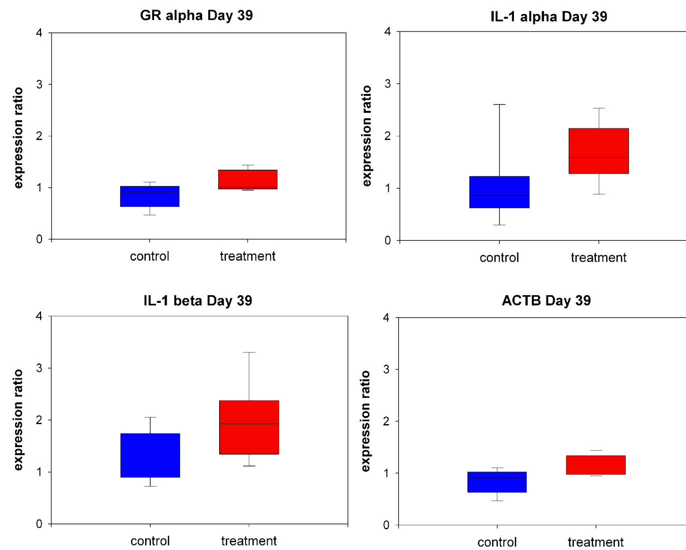

Figure 5: Significant regulations for GRα (A), IL-1α (B), IL-1β (C) and ACTB (D) between control and treated samples after 39 days of treatment.

The number of quantified genes is yet too less to draw conclusions on the different pathways, but anyhow first physiological interpretations can be made and genes that might act as potential biomarkers could be identified.

The steroid receptors GRα and ERα show an up-regulation in the treatment group compared to the control. GRα is up-regulated at day 16 and day 39, whereas ERα is only up-regulated at day 16. Trenbolone acetate has an antiglucocorticoid effect via binding to GR [79-81]. It is already shown that steroid hormones influence the mRNA expression of their receptors in different tissues [82-85]. The applied hormone combination acts via GRα and ERα. The up-regulation of these receptors indicates stimulation of the expression of these receptors in white blood cells.

The interleukins IL-1α and IL-1β are up-regulated. IL-1α is up-regulated at day 16 and day 39, whereas IL-1β is only regulated after 39 days of treatment. IL-1α and IL-1β are produced by macrophages, monocytes and dendritic cells. During infection they induce the release of other cytokines. The expression of IL-1β can be induced by IL-1α. This could be an explanation why IL-1α is up-regulated after 16 days of treatment whereas IL-1β is only up-regulated after 39 days of treatment [86, 87].

PCA is a technique used to reduce multidimensional data sets to lower dimensions for analysis. This statistical method was used to determine whether there is a clustering between control and treatment group. Figure 6 was obtained by plotting all samples of the two groups in the different time points by their two principal components obtained from the 11 regulated genes. Each group was marked by a color. Blue crosses represent samples of the control group and red triangles show the samples of the treatment group. At day 2

and 16 of treatment it can be observed that both groups arrange together and that a difference between control and treatment group can be monitored.

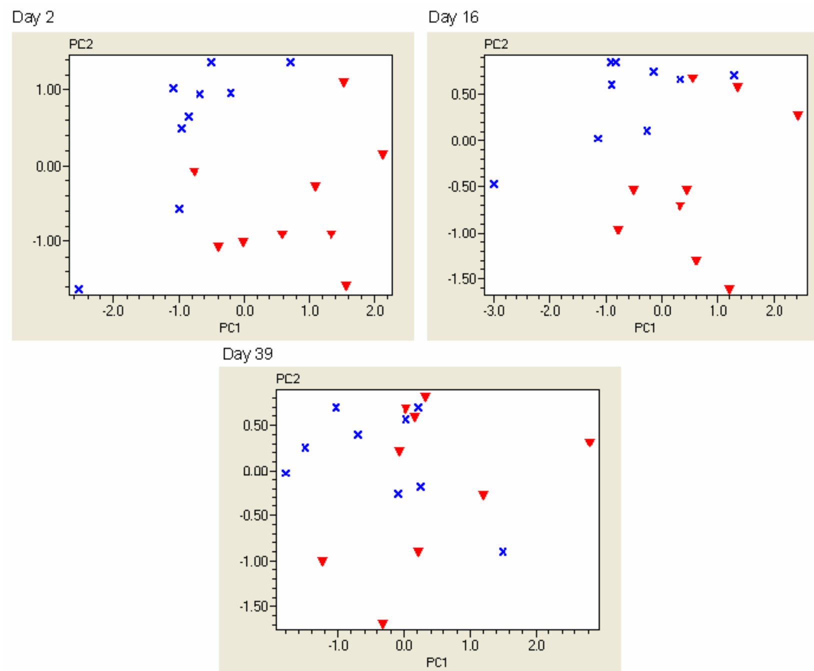

Figure 6: PCA for the eleven regulated genes GR-α, ER-α, Fas, IL-1α, IL-1β, IL-6, MHCII, CK, MTPN, RBM5 and Actin-β at the three different treatment time points. Animals of the control groups are represented by blue crosses and animals of the treatment group are represented by red triangles.

Vaginal smear

The steroid receptor ERα showed a significant down-regulation after two days of treatment (p=0.046). Hormones regulate the concentrations of their receptor proteins either by regulating the transcription of the receptor gene or by regulating the stability of the receptor mRNA [88]. The observed down-regulating effect of estrogens on the estrogen receptor was already reported for vaginal cells of mice and rats [89, 90].

The pro-inflammatory interleukins IL-1α (p=0.016) and IL-1β (p=0.005) were both down-regulated after 39 days of treatment.

The keratinization factor CK8 (p=0.003) was significantly down-regulated after two days of treatment. The growth factors FGF7 (p=0.009), EGF (p=0.005), EGFR ($p=0.5\times10^{-4}$), TGFα ($p=0.5\times10^{-3}$), IGF-1R (p=0.007) and LTF (p=0.031) were significantly regulated, whereas EGF, TGFα, IGF-1R were down-regulated at day 2 and FGF7 and EGFR were up-regulated after 16 days and LTF was up-regulated after 39 days of treatment.

At the end of the estrous cycle estrogen levels are high. At this phase vaginal epithelium proliferates, the epithelial cells keratinize and get detached [91].

The keratinization factor CK8 is preferentially expressed in epithelial cells, e.g. in vaginal epithelium. In mice it was already shown that estrogens down-regulate the mRNA expression of this factor [92]. Factors that are involved in the stimulation of the proliferation of epithelial cells are the growth factors FGF7, EGF and EGFR [93-95]. FGF7 and EGF stimulate epithelial growth in vaginal epithelium in mice [96-98]. Both factors were up-regulated after 16 days of treatment. The expression of EGFR was down-regulated after two days of treatment. The effect of estrogens on mRNA expression of these three factors was already shown in mice vaginal epithelial cells [99-102]. The regulation of the growth factors IGF-1R and LTF also goes in line with effects of estrogens that could already be shown in mice. Miyagawa et al. (2004) reported, that the mRNA expression of members of the IGF family is regulated by diethylstilbestrol, a synthetic nonsteroidal estrogen [103]. In this study the down-regulating effect of estrogens on IGF-1R could also be observed. Sato et al. (2004) demonstrated that neonatal exposure of mice with diethylstilbestrol results in an up-regulation of EGF and LTF [104, 105].

It is already known that estrogens stimulate LTF mRNA expression in uterine tissue [65, 106] and that LTF is present at various stages of the estrous cycle in human uterus and vaginal epithelium [65, 107, 108]. This study shows that LTF mRNA expression is increased by estrogen treatment in the bovine vaginal epithelium. The expression of LAP, another defensin was not influenced by the treatment.

Most effects on mRNA expression shown in this study were already obvious in mice and rats. This indicates that the effect of estrogen on the vaginal epithelium is highly conserved. In the 1950s Edgren et al. (1957, 1959) reported that androgens inhibit vaginal effects of estrogens like keratinization of the vaginal epithelium [109, 110]. This study indicates that trenbolone acetate does not show this antagonistic effect.

The oncogene c jun showed a down-regulation at day 2 (p=0.005). Furthermore ACTB (down-regulation at day 2, p=0.007) and UB3 (down-regulation at day 2, p=0.018, and day 16, p=0.001) were significantly regulated. The expression ratios of all regulated genes are listed in table 7.

Table 7: Significant expression changes. Fold regulations between treatment and control group of the significant regulated genes at the three treatment time points. Green background indicates down-regulation and red background indicates up-regulation of the genes.

Gene Group	Gene	Day 2	Day 16	Day 39
Steroid receptors	ERα	0.59		
Keratinization factors	CK8	0.42		
Growth factors	FGF7		2.6	
	EGFR	0.36		
	EGF		2.79	
	IGF-1R	0.63		
	TGFα	0.25		
	LTF			4.35
Interleukins	IL-1α			0.34
	IL-1β			0.2
Oncogenes	c jun	0.61		
Others	ACTB	0.46		
	UB3	0.64	0.31	

The second aim of this study was to investigate whether the observed changes of mRNA expression could act as biomarkers to develop a screening method for the combination of trenbolone acetate plus estradiol.

PCA is a technique used to reduce multidimensional data sets to lower dimensions for analysis. This statistical method was used to determine whether there is a clustering between control and treatment group. Figure 7 was obtained by plotting all samples of the two groups in the different time points by their two principal components obtained from the 13 regulated genes. Some genes showed no significant regulation, but showed a trend to be regulated ($p<0.1$) Therefore PCA was also done by plotting all samples of the two groups in the different time points by their two principal components obtained from all 27 measured candidate genes (Figure 8).

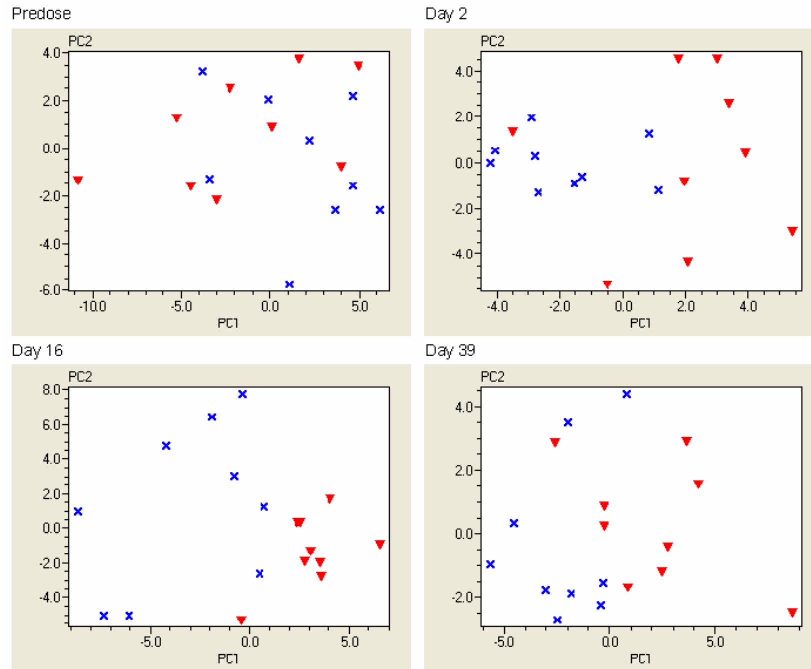

Figure 7: Principal components analysis (PCA) for the thirteen significantly regulated genes at the four different sampling time points. Animals of the control groups are represented by blue crosses and animals of the treatment group are represented by red triangles.

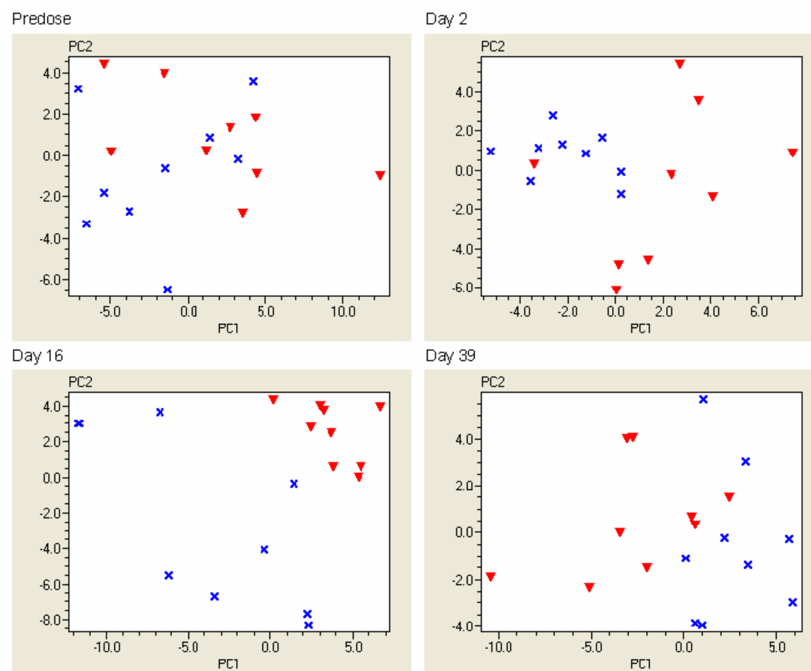

Figure 8: Principal components analysis (PCA) for all 27 measured candidate genes at the four different sampling time points. Animals of the control groups are represented by blue crosses and animals of the treatment group are represented by red triangles.

At all three treatment time points both groups arrange together and a difference between control and treatment group can be monitored. Before treatment the groups show no difference in gene expression of analyzed target genes. This effect is better visible using all 27 quantified genes.

Another biostatistical method to visualize wether the groups arrange together is Hierarchical Cluster Analysis. To verify if the effect observed by PCA is also visibly by using this method, hierarchical clustering was done with the data of the day 16 samples obtained from all measured genes (Figure 9). The dendogram shows a clear separation between the two groups by showing two main branches. The one above only represents control samples. The other one represents treatment samples exept of sample control 6. Performed as treatment screening this sample would be a false positive one.

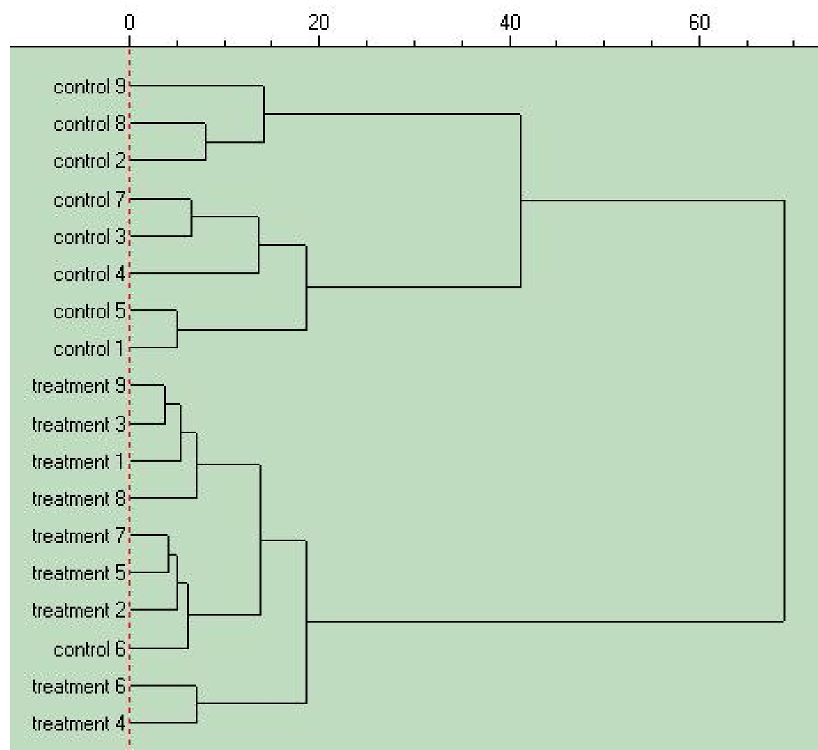

Figure 9: Dendogram for all 27 measured candidate genes at sampling time point day 16.

3.2 Pour on anabolics study in veal calves

RNA Integrity

Good RNA quality is important for the overall success of RNA based analysis methods like real time RT-qPCR [75, 76, 111, 112]. The RNA degradation level was determined

using the lab-on-a-chip technology of the Agilent Bioanalyzer (Agilent Technologies). The RIN value of the blood samples was 8.5 ± 0.4 (mean ± SD) indicating well intact RNA.

Primer testing

Primer pairs of 32 genes were successfully used in RT-qPCR analysis to get single peaks and uniform melting curves.

RT-qPCR results and data analysis

The carrier control group (CC) showed no significant differences in gene expression compared to the untreated control group and hence the two groups were layed together as one control group of 10 animals for further analyses.

There were no significant differences in the expression of measured target genes 2 and 7 days after treatment start. The steroid receptors ERα, ERβ and GRα were significantly down-regulated in the T3 group, whereas GRα was regulated 14 (p=0.006), ERβ 63 (p=0.031) and ERα 63 (p=0.054) and 91 (p=0.003) days after treatment start. The apoptosis regulators FasL and TNFα were significantly down-regulated in the T3 group, whereas FasL was only regulated 63 days (p=0.050) and TNFα showed a significant regulation 14 (p=0.0005), 21 (p=0.002) and 63 (p=0.004) days after treatment start. The pro-inflammatory factor IL-12B was significantly down-regulated in the T3 group 63 days after treatment start (p=0.010). The transcription factor NFκB showed a significant down-regulation in the T1 (p=0.011) and the T3 group (p=0.012) 63 days after treatment start. CD4 was significantly down-regulated in the T3 group 35 days after treatment start (p=0.025). ACTB was significantly up-regulated in the T3 group 91 days after treatment start (p=0.035) and UB 3 was significantly down-regulated in the T3 group 35 days after treatment start (p=0.045). Table 8 shows the x-fold regulations of all significantly regulated genes at each time point.

Table 8: x-fold regulations of all significantly regulated genes at all timepoints. Green background indicates down-regulation and red background indicates up-regulation of the genes.

Gene group	Gene	Treatment Group	Day 14	Day 21	Day 35	Day 63	Day 91
Steroid hormone receptors	ERα	T3				0.75	0.75
	ERβ	T3				0.52	
	GRα	T3	0.46				
Apoptosis regulators	FasL	T3				0.64	
Pro-inflammatory factors	TNFα	T3	0.66	0.83		0.76	
	IL-12B	T3				0.43	
Transcription factors	NFκB	T1				0.81	
	NFκB	T3				0.73	
CD Antigen	CD4	T3			0.62		
Others	ACTB	T3					1.23
	UB 3	T3			0.76		

The number of quantified genes was yet too less to draw conclusions on the different pathways, but anyhow first physiological declarations can be made and genes that could act as potential biomarkers could be identified.

The mRNA expression of the steroid receptors ERα, ERβ and GRα was significantly down-regulated. It is already shown that steroid hormones influence the mRNA expression of their receptors in different tissues [113-116], either by regulating the transcription of their receptor gene or by regulating the stability of the receptor mRNA [117].

The significantly down-regulated apoptosis factors TNFα and FasL belong to the TNF Family [118] and induce apoptosis by binding to the death receptors TNFR1, TNFR2 or Fas. The down-regulation of these apoptosis regulators suggest that the immune response is suppressed by the treatment with the used hormone cocktail. This effect seems to be induced by testosterone, because it has already been proven that testosterone has a suppressive effect on the immune system [119, 120].

The pro-inflammatory factor IL-12B was significantly down-regulated in the T3 group 63 days after treatment start. IL-12B – a subunit of IL12 – is mainly produced by monocytes, dendritic cells and activated macrophages. It promotes IFNγ production by CD4 positive T-cells and stimulates proliferation and cytotoxic activity of T-cells and natural killer cells.

Differences in weight gain

The CC group showed no significant differences in weight gain and carcass weight compared to the untreated control group and hence the two groups were layed together as one control group of 10 animals for further statistical analyses.

No differences in weight gain between the two treatment groups could be observed 28 days after treatment start. 63 days after treatment start the difference in weight gain between the control and the T3 group is not significant but a trend is visible p<0.1. At the end of the trial (day 91) the difference in weight gain between the control and the T3 group was significant, whereas there was no mentionable difference between the control and the T1 group. Regarding carcass weight, it can be observed that the T1 group shows a trendly increase compared to the control group (p = 0.1) and difference of the T3 group compared to the control group increased significantly (p = 0.01).
Differences in weight gain and carcass weight are shown in figure 10 and 11.

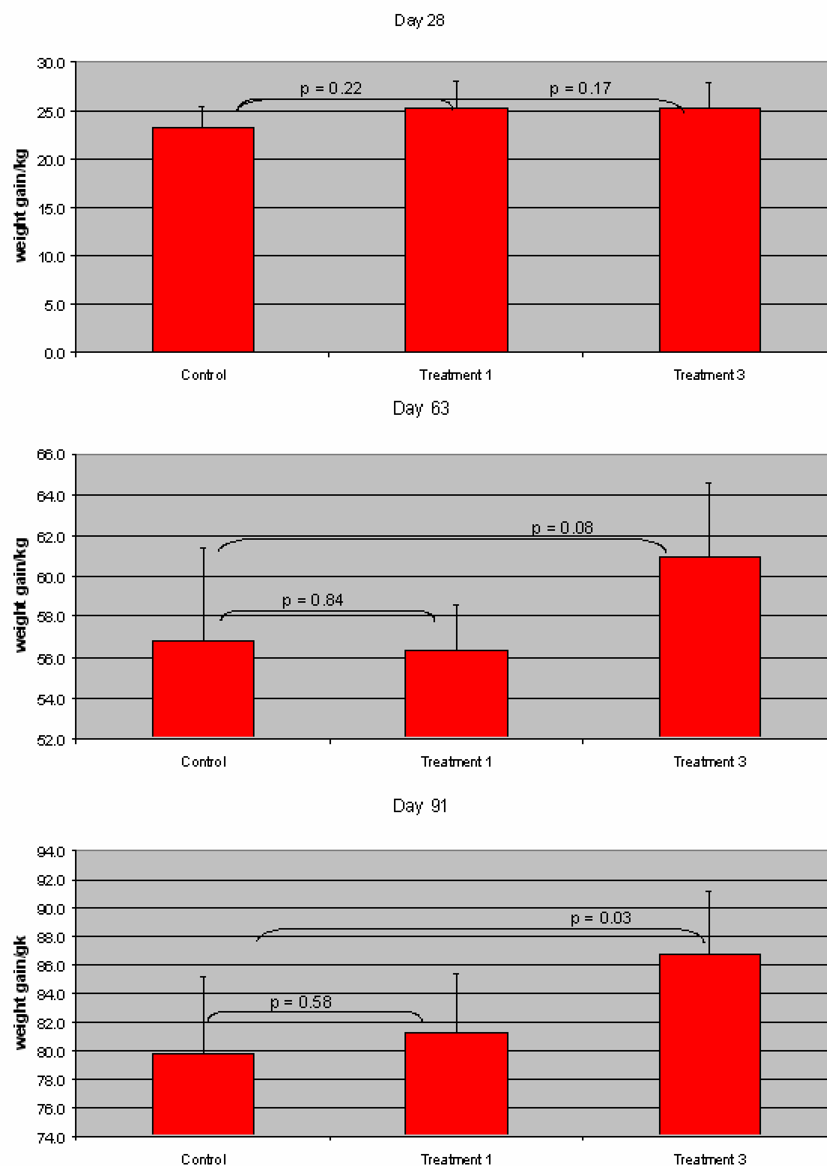

Figure 10 : Differences in live weight gain after 28, 63 and 91 days of treatment

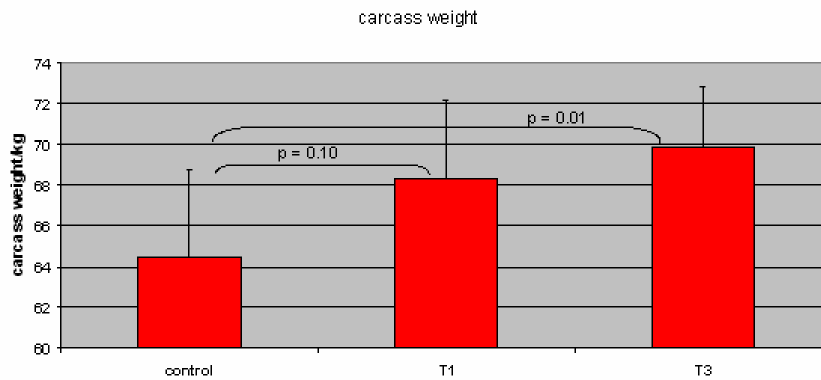

Figure 11 : Differences in carcass weight

The trendly differences in weight gain in the T3 group occur primarily 63 days after treatment start. This is in line with the fact that most differences in gene expression could be observed 63 days after the beginning of treatment. The treatment with anabolic hormones via pour on seems to have no significant effect without being repeated. Another conclusion is that a hormone depot is built which releases the hormones stepwise. This goes in line with observations done by Rattenberger et al. (1993) who could measure hormone residues of diethylstilbestrol and nortestosterone in urine of calves treated with these hormones via pour on even 138 days after treatment [121].

3.3 SARM Study on *Macaca fascicularis*

RNA Integrity

The mean (±SD) RIN value of the blood samples were 7.5 (± 4.8) at predose, 8.5 (± 5.0) on day 16 and 7.7 (± 4.2) at day 90 indicating well intact RNA.

Primer testing

Primer pairs of 40 genes were successfully used in RT-qPCR analysis to get single peaks and uniform melting curves.

RT-qPCR results and data analysis

In this study changes of gene expression in blood cells caused by treatment with LGD2941 or testosterone were evaluated in order to describe physiological effects and to find potential biomarkers for the treatment with AR ligands.

Significant down-regulation of gene expression of the treatment groups compared to the control group could be identified for IL-15 ($p=0.0093$) and TNFR2 ($p<0.0001$) after 16

days (Figure 12) and for IL-15 (p=0.0498), CD30L (p=0.0435), Fas (p=0.0032), TNFR1 (p=0.0308) and TNFR2 (p<0.0001) after 90 days of treatment. Significant up-regulation of gene expression of the treatment groups compared to the control group could be observed for IL-12B (p=0.0240) after 90 days of treatment (Figure 13, 14).

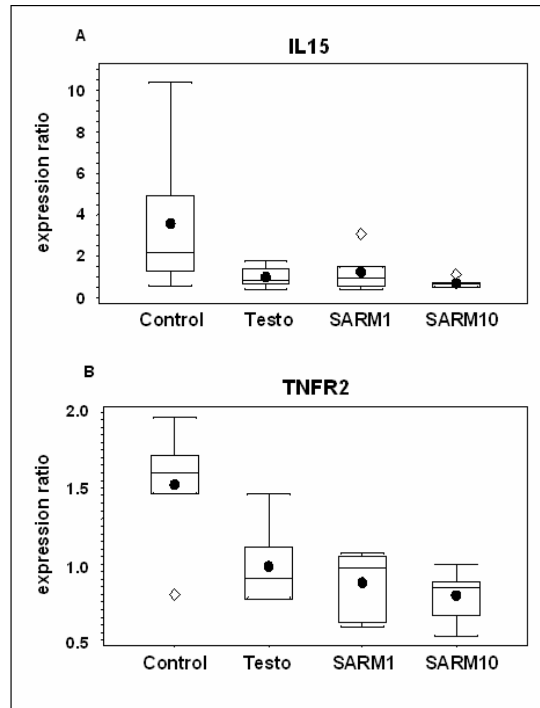

Figure 12: Significant regulation for IL-15 (A) and TNFR2 (B) between control and treated samples after 16 days of treatment.

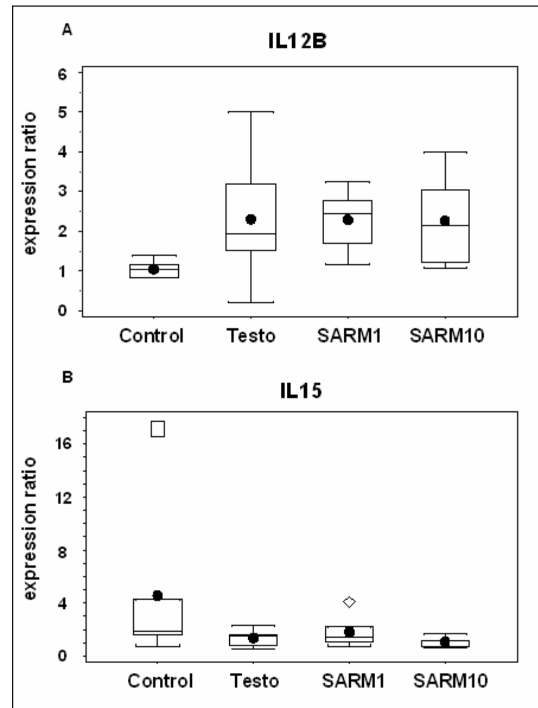

Figure 13: Significant regulation for the proinflammatory interleukins IL-12B (A) and IL-15 (B), between control and treated samples after 90 days of treatment.

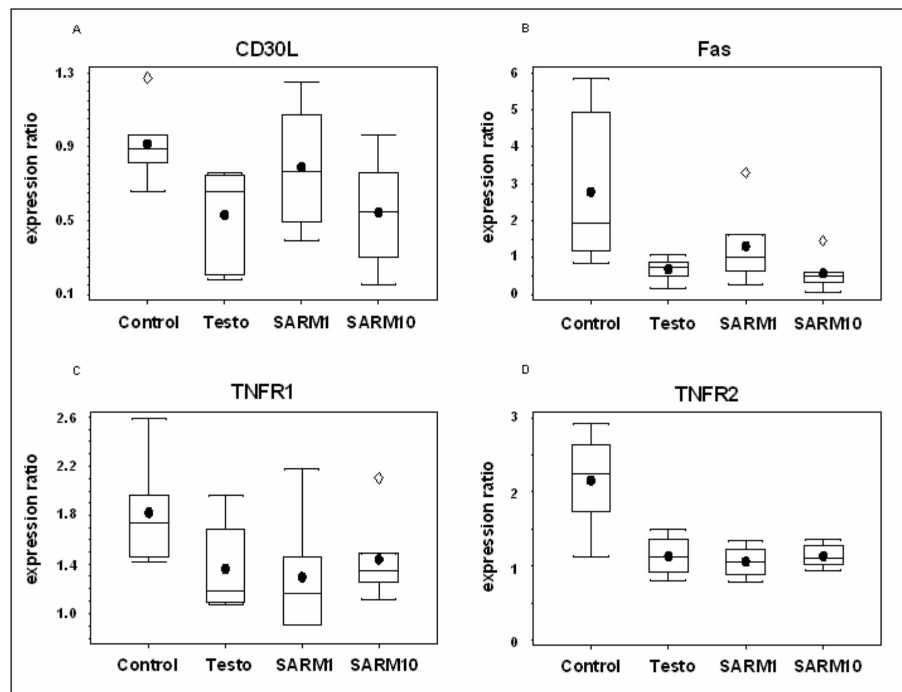

Figure 14: Significant regulation for the apoptosis regulators CD30L (A), Fas (B), TNFR1 (C) and TNFR2 (D) between control and treated samples after 90 days of treatment.

Regarding the Box-whisker plots it can be monitored that the statistical variance in the control group is higher than in the treatment groups. The reason for this could be the natural variability of the non induced expression in each studied subject. Suppression of gene expression by an external stimulus like treatment with testosterone or the SARM reduces natural variability of gene expression.

The main physiological effect that could be observed in this study is the down-regulation of various apoptotic marker genes in all three treatment groups. This is shown by the significant regulation ($p<0.05$) of the apoptosis receptors Fas, TNFR1, TNFR2 and the apoptosis ligand CD30L. All regulated apoptosis factors belong either to the TNF Family (CD30L) or to the TNF-Receptor Family (TNFR1, TNFR2, Fas) [122]. The down-regulation of these apoptosis regulators suggest that the immune response is suppressed by the treatment with testosterone and the SARM. This is consistent with the fact that testosterone has a suppressive effect on the immune system [123, 124]. If the physiological effects of testosterone and the SARM are compared it became obvious that the SARM is similarly active to natural androgens.

PCA is a technique used to reduce multidimensional data sets to lower dimensions for analysis. Figure 15 was obtained by plotting all samples of the four treatment groups by their two principal components obtained from the six responder genes. Blue dots represent samples of the control group, light green dots show the testosterone group, olive dots represent the SARM1 group and the red dots display the SARM10 group. A distinct control group can be seen, showing that there was a multitranscriptional response to the treatment by any of the three drugs. In addition, the SARM1 neighbors to the control group, creating thus a transition to the Testosterone group and the SARM10 group.

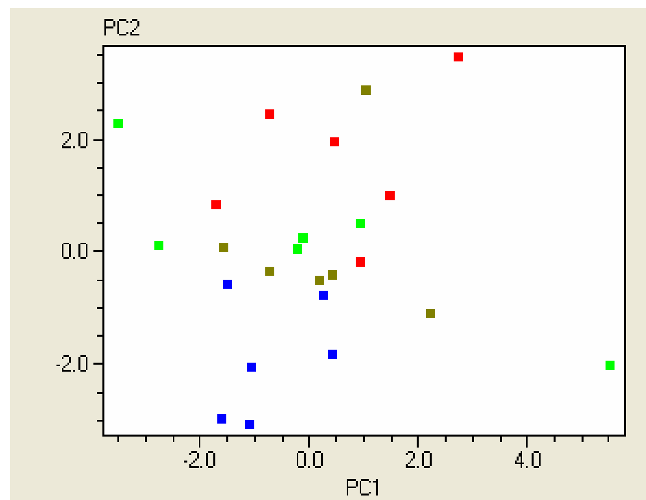

Figure 15: PCA for the six regulated genes IL-12B, IL-15, CD30L, Fas, TNFR1 and TNFR2 in the control group (blue dots) the testosterone treated group (light green dots) the low dosed SARM group (olive dots) and the high dosed SARM group (red dots).

To verify if there is any correlation of the different regulated genes, the six responder genes were clustered by PCA (figure 16). Red dots show apoptosis regulators and black spots display the interleukins. The TNF receptors cluster very closed together, so that the two spots representing these factors are difficult to separate. This indicates that the two TNF receptors might be coregulated. The other genes do not show any coherence.

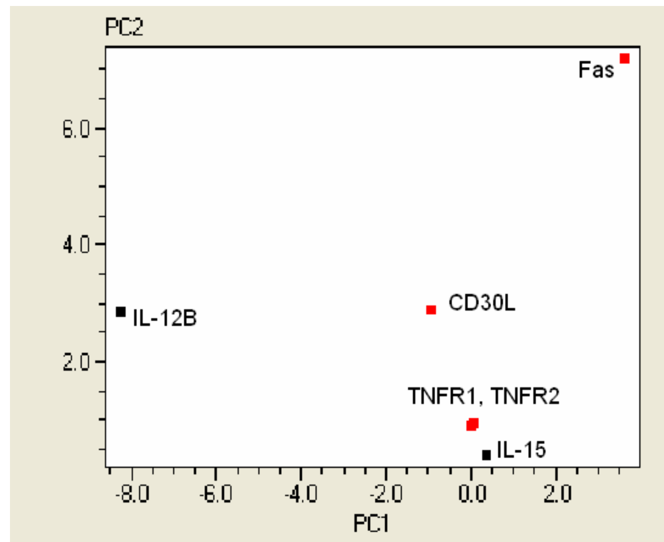

Figure 16: PCA for the regulated genes in all four groups. Black spots show the interleukins and red spots show the apoptosis regulators

The second aim of this study was to find potential biomarkers for the use of the SARM. Regarding PCA it can be postulated that the regulated genes found in this study can act as first biomarker candidates for the development of a screening pattern in whole blood.

4 Conclusions and Perspectives

In all three animal trials included in this thesis the potential of gene expression analysis for developing a new screening method to trace the use of anabolic steroid hormones is examined. Combined with biostatistical methods, like PCA or hierarchical cluster analysis this approach seems to be auspicious.

Although the quality of RNA obtained from bovine vaginal smear is poor, gene expression data in combination with PCA or hierarchical cluster analysis show promising results for the development of potential gene expression biomarkers. Both biostatistical methods show a clear clustering of the treatment groups. The disadvantage of this matrix is, that vaginal smear is only available from female animals. Regarding this, blood samples display a better matrix, because blood can be taken from the living animal independent of gender.

In all three animal trials, changes in gene expression could be quantified in blood samples. Comparing the results obtained from the bovine animal trials it could be observed that only a few genes show expression changes in both studies. Genes like the steroid receptors ERα and GRα are regulated in both experiments but are regulated in different directions. The only gene whose mRNA expression is similarly regulated in both studies is ACTB. Comparing the results obtained from blood samples of the bovine trials and the SARM study it could be shown that only Fas and IL-12B are regulated in both systems, whereas the genes are regulated in different directions. Figure 17 presents an overview of all regulated genes obtained from the three animal trials.

These results indicate that the influence of anabolic steroid hormones on gene expression in blood is species specific and dependent on breed, age, application method and possibly the applied hormone.

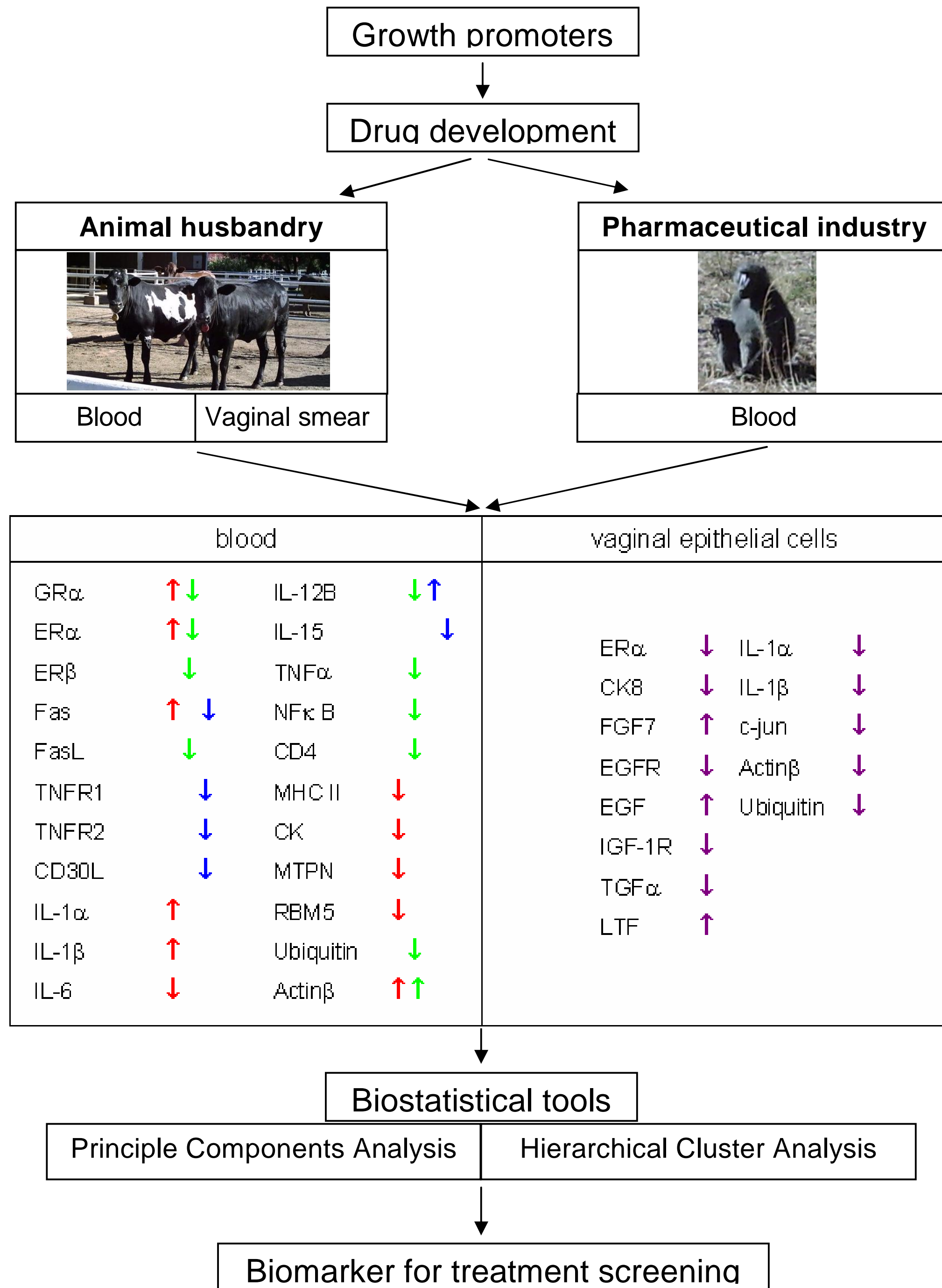

Figure 17: Schematic description of the regulated genes obtained in this thesis.

The direction of regulation is described by arrows. ↓ describes down-regulation and ↑ describes up-regulation. The different tissues and studies are marked by color. Red arrows describe results obtained from blood samples in the study on Nguni cattle, green arrows describe results obtained from blood samples of the pour on study, blue arrows describe results obtained from blood samples of the SARM study and violet arrows describe results obtained from vaginal smear samples of the study on Nguni cattle.

In all three studies the method of PCA was employed to prove if a treatment pattern is visible. Using results obtained from blood samples in the trial on Nguni heifers and the SARM study, clustering of the treatment groups is visible.

Regarding the pour on trial, most changes in gene expression are present 63 days after treatment start, but only in the three times treated group. Obtained gene expression changes are minimal so that biostatistical tools like PCA or hierarchical cluster analysis do not show any successful clustering of the animals.

Although weight gain results of the pour on trial indicate that application of anabolic hormones via pour on show the intended anabolic effect, the use of anabolic steroid hormones by this application method seems not to be detectable on the level of gene expression in blood. This indicates first problems of using gene expression analysis for the development of a new method to screen for a broad range of anabolic agents independent of their way of application. Nevertheless, to determine if the use of gene expression changes to develop a biomarker pattern for the use of anabolic agents is still promising, other tissues, like liver, kidney or different hormone responsive muscles have to be taken into account.

To develop a screening method by regarding physiological effects of anabolic hormones in blood, the additional use of other *omic* technologies like proteomics or metabolomics will be a promising way. From the literature it is known that the blood levels of different proteins, like IGF-1, IGF-1BP3, GH, HDL-C or LDL-C are changed by the treatment of anabolic agents. Regarding metabolomics, most is known about the influence of β-agonists on blood metabolites. Levels of Nτ-methylhistidine, creatinine, non-esterified fatty acids or natural catecholamines are influenced by the use of β-agonists [38]. 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 [38].

The combination of transcriptomics, proteomics and metabolomics with special biostatistical methods, like hierarchical cluster analysis, canonical correlation analysis and linear or multiple discriminant analysis will be a new perspective for developing a new screening method to trace the abuse of anabolic steroid hormones [38].

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Scientific Communication

Original Publications

M.W. Pfaffl, S. Fleige, I. Riedmaier
Validation of lab-on-chip capillary electrophoresis systems for total RNA quality and quantity control
Biotechnology and Biotechnological Equipment, 2008, 22/3.

I. Riedmaier, C. Becker, M.W. Pfaffl, H.H.D. Meyer
The use of *omic* technologies for biomarker development to trace anabolic hormone functions
Journal of Chromatography A (2009), doi:10.1016/j.chroma.2009.01.094

I. Riedmaier, A. Tichopad, M. Reiter, M.W. Pfaffl, H.H.D. Meyer
Influence of testosterone and a novel SARM on gene expression in whole blood of *Macaca fascicularis*
Journal of Steroid Biochemistry and Molecular Biology, 2009, 114: 167-173

I. Riedmaier, A. Tichopad, M. Reiter, M.W. Pfaffl, H.H.D. Meyer
Identification of potential gene expression biomarkers for the surveillance of anabolic agents in bovine blood cells
Analytica Chimica Acta, 2009; 638: 106-113

I. Riedmaier, M. Reiter, A. Tichopad, M.W. Pfaffl, H.H.D. Meyer
The potential of bovine vaginal smear for biomarker development to trace the misuse of anabolic agents
Submitted: “The Analyst”

Oral Presentations:

I. Riedmaier, C. Becker, M.W. Pfaffl, H.H.D. Meyer
The use of transcriptomics for biomarker development to trace anabolic hormone functions
4th International qPCR Symposium, Technische Universität München, 09.03.-13.03.2009, Freising-Weihenstephan, Germany.

Poster Presentations:

I. Riedmaier, A. Tichopad, M. Reiter, M.W. Pfaffl, H.H.D. Meyer
Influence of testosterone and a novel SARM on gene expression in whole blood of *Macaca fascicularis*
18th Symposium of the Journal of Steroid Biochemistry and Molecular Biology, 18.09.-21.09. 2008, Seefeld in Tirol, Österreich

I. Riedmaier, M. Reiter, A. Tichopad, M.W. Pfaffl, H.H.D. Meyer
Identification of potential gene expression biomarkers in bovine vaginal smear after application of the anabolic combination trenbolone acetate plus estradiol
18th Symposium of the Journal of Steroid Biochemistry and Molecular Biology, 18.09.-21.09. 2008, Seefeld in Tirol, Österreich

I. Riedmaier, M. Bergmaier, M.W. Pfaffl
Comparison of two available platforms for the determination of RNA quality
4th International qPCR Symposium, Technische Universität München, 09.03.-13.03.2009, Freising-Weihenstephan, Germany.

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Appendix

Appendix I:

M.W. Pfaffl, S. Fleige, I. Riedmaier

Validation of lab-on-chip capillary electrophoresis systems for total RNA quality and quantity control

Biotechnology and Biotechnological Equipment, 2008, 22/3.

Appendix II:

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Appendix III:

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Journal of Steroid Biochemistry and Molecular Biology, 2009, 114: 167-173

Appendix IV:

I. Riedmaier, A. Tichopad, M. Reiter, M.W. Pfaffl, H.H.D. Meyer

Identification of potential gene expression biomarkers for the surveillance of anabolic agents in bovine blood cells

Analytica Chimica Acta, 2009; 638: 106-113

Appendix V:

I. Riedmaier, M. Reiter, A. Tichopad, M.W. Pfaffl, H.H.D. Meyer

The potential of bovine vaginal smear for biomarker development to trace the misuse of anabolic agents

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Appendix I

VALIDATION OF LAB-ON-CHIP CAPILLARY ELECTROPHORESIS SYSTEMS FOR TOTAL RNA QUALITY AND QUANTITY CONTROL

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ABSTRACT

Purity and good RNA quality are important elements for the overall success of RNA based analysis methods like microarrays and real time qRT-PCR. There are two commercially available automated systems – the Experion (Bio-Rad Laboratories) and the 2100 Bioanalyzer (Agilent Technologies) – that provide both RNA sample quality and quantity analysis. In this study different aspects like the reproducibility and sensitivity of both systems were analyzed by determining the total RNA quality and quantity extracted from various bovine tissues. Regarding quantitation, the Experion is more sensitive than the 2100 Bioanalyzer. Both systems overstate the concentration by 19-29% compared to the photometric values. For RNA quality determination, both systems show highly comparable reproducibility. With the RNA integrity number (RIN) the 2100 Bioanalyzer offers an additional opportunity to quantify the RNA quality.

Keywords: RNA integrity, RNA quality, RNA quantity, transcriptomics, lab-on-chip, capillary electrophoresis

Introduction

Methods for Gene Expression measurements like microarray technology and quantitative real time RT-PCR (qRT-PCR) require high quality RNA (1, 3, 4, 5). The purity of RNA is normally assessed by its $OD_{260\ nm}/OD_{280\ nm}$ ratio (2) but using this tool no information is given according RNA integrity. For decades, scientists have gained essential data about their nucleic acid RNA samples from the use of agarose gel-based electrophoresis. Gel electrophoresis, the movement and separation of charged particles in response to an electric field, results in an unparalleled and irreproducible resolution of RNA molecules. However, the process is composed of a series of manual steps that require numerous pieces of equipment, various reagents, and several hours to gain the information needed from sample separations. Since this traditional process requires significant time and manual labour, automation has been a key desire of scientists. The Experion (Bio-Rad Laboratories, Hercules, CA) and the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) system apply innovative micro-fluidic separation technology to both automate and accelerate this process.

The lab-on-chip is an integrated part of the micro-fluidic system, working together with specialized computer-controlled instrumentation used to manipulate the timing and sequence of the processes designed into the chip. As a result, these systems inherently generate accurate and reproducible data. Nevertheless, chip design must provide optimal channel and sample well dimensions, and system design must provide precise control over temperature, flow rates, and sample injection and separation voltages, amongst good laboratory praxis, in order to produce optimal results. When micro-fluidic chips are used for electrophoresis, the process is very similar to that of traditional gel-based electrophoresis. The main difference is that micro-fluidics enables the miniaturization and combination of multiple steps of gel-based electrophoresis – separation, staining, containing, imaging, and even basic data analysis – into a single automated process.

In this study we determined the RNA quality and quantity of different bovine tissues by using the Experion (Bio-Rad Laboratories) and the 2100 Bioanalyzer (Agilent Technologies). The intention was to investigate the comparability and validity of the results delivered by both lab-on-chip systems. As the policy for these two devices does not differ, the analysis of all samples should lead to similar results. The appearance of differences in the achieved results has to be borne in mind when interpreting data from these two devices.

Materials and Methods

Total RNA

Total RNA purification was performed by an in-house standardized phenol-based extraction methods using TriFast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. Total RNA was extracted from various bovine tissues, and was carried out in RNAse-free environment. To show tissue independent quality and quantity studies, eleven different bovine tissue sources were under investigation. Purified total RNA was eluted in the column using RNAse-free water (Eppendorf, Hamburg, Germany). First RNA integrity was verified in triplicates by UV measurement, using the BioPhotometer (Eppendorf) and additionally the NanoDrop 1000 (Peqlab). Only samples with an $OD_{260\ nm}/OD_{280\ nm}$ absorption ratio higher than 1.85 were used for the further investigations.

Hardware

For microcapillary electrophoresis measurement, the Experion system was used in conjunction with the Experion RNA

StdSens kit (Bio-Rad) and the 2100 Bioanalyzer with the RNA 6000 Nano LabChip analysis kit (Agilent Technologies) and the 6000 RNA ladder (Ambion, Austin, TX). Total RNA samples and ladders were prepared according to the protocols provided in the instruction manuals of the Experion RNA analysis kit and the Agilent RNA 6000 LabChip kit. To prevent systematic handling errors one single user performed the assays on both micro-capillary electrophoresis systems in parallel. In the project the comparability of the results of both analytical systems were analyzed with two different total RNA concentrations: 50 ng/µl or 200 ng/µl. The RNA Integrity Number (RIN) software algorithm of the 2100 Bioanalyzer permits the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact (6).

Sample degradation

The first sub-project was conducted concentrating on different RNA degradation levels. To get RNA samples with different degradation levels, but with the identical transcriptome and mRNA distribution, total cellular RNA was degraded by irradiation with ultraviolet (UV) light, as described earlier by Fleige et. al (5). Depending on the type of tissue each sample was placed under UV-lamp for a different period of time up to 1.5 h. Intact and degraded samples from identical tissue extraction, containing the identical transcriptome, were mixed in various ratios to generate a linear degradation gradient.

Statistical analysis

Descriptive statistics were generated using the Sigma Stat software, version 3.0 (SPSS Inc, Chicago, IL, USA). Mean, standard deviation (SD) and coefficient of variation (CV) between and within groups of samples were determined. Comparative statistical analyzes between groups were completed, using non-parametric statistical tests: Mann-Whitney Rank Sum Test. Coefficients were recorded when significant at a minimum of $p<0.05$. Higher significance levels were considered when available. Data were statistically processed in Sigma Plot 8.0 (SPSS, Inc.) and Excel (Microsoft, Seattle, US).

Results and Discussion

In this study the RNA quality and quantity of different bovine tissues was determined by the Experion (Bio-Rad) and the 2100 Bioanalyzer (Agilent). By analyzing the same samples in parallel the comparability of both systems is given. By mixing an inter RNA sample with a qualitative degraded version of the same sample it was possible to impair the RNA quality factitiously (4). The creation of different mixing ratios made it possible to alter the samples in nuances from degraded to integer RNA quality. Therefore, it was possible to analyze the trend of the measurements of degraded RNA.

Ribosomal 28S/18S ratio

RNA integrity was first assessed by resolving the 28S and 18S ribosomal RNA band comparing both lab-on-chip systems. It is stated, that the ribosomal 28S/18S ratio plays an important role in determining the level of sample degradation in gel electrophoresis. Ratio analysis was done in total on 180 different total RNA sample profiles. Ratio data from 16 samples was not obtained due to device problems during the runs (8.9%). From one selected bovine tissue 12 RNA degradation levels were assessed using both separation systems. The results shown in figure 1 are from repeated determinations by using the Experion and a threefold determination with the 2100 Bioanalyzer. Furthermore, the Experion 28S/18S ratios are overall lower than the 2100 Bioanalyzer values. Both the 2100 Bioanalyzer and Experion data were also correlated to the RIN, because this mirrored RNA quality, notwithstanding this feature is not available in the Experion software. The graphs in figure 1 demonstrate the correlation between the ribosomal 28S/18S ratio and the degree of RNA degradation. As expected, the 28S/18S ratio rises with the increasing of sample quality. This applies to both systems. The comparison of the trend-lines leads to the conclusion that the slopes of the 2100 Bioanalyzer data are greater than those of the Experion. This trend is reflected in all regressions performed on various tissues (data not shown) and in selected single run comparisons, e.g. shown in figures 2a – 2c with total RNA extracted from bovine corpus luteum and caecum.

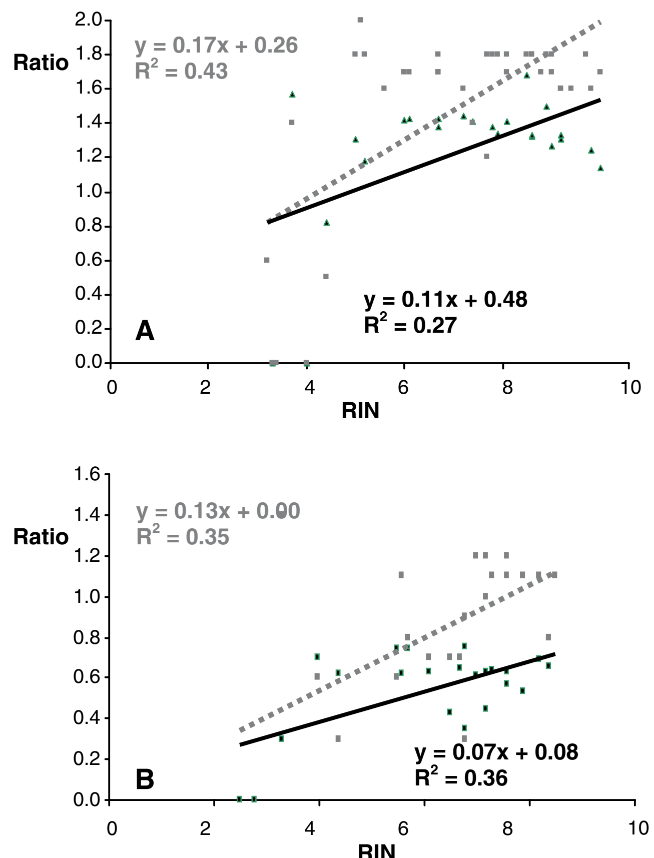
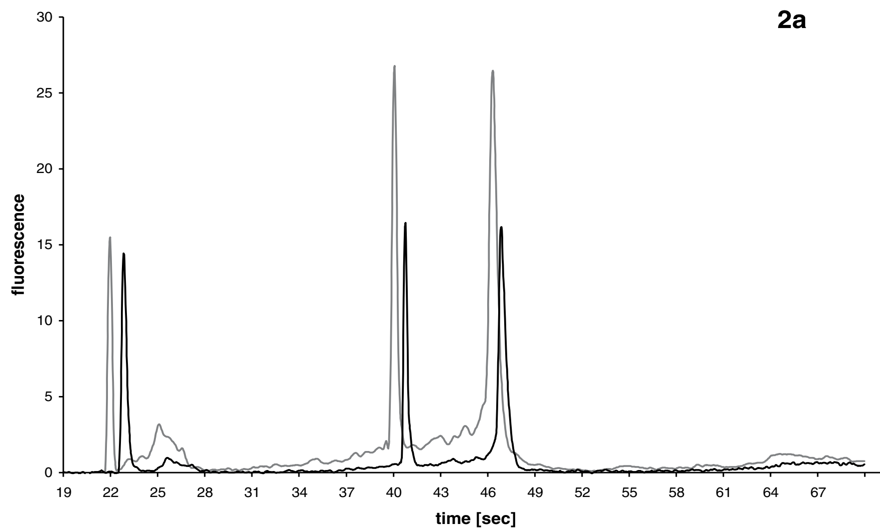
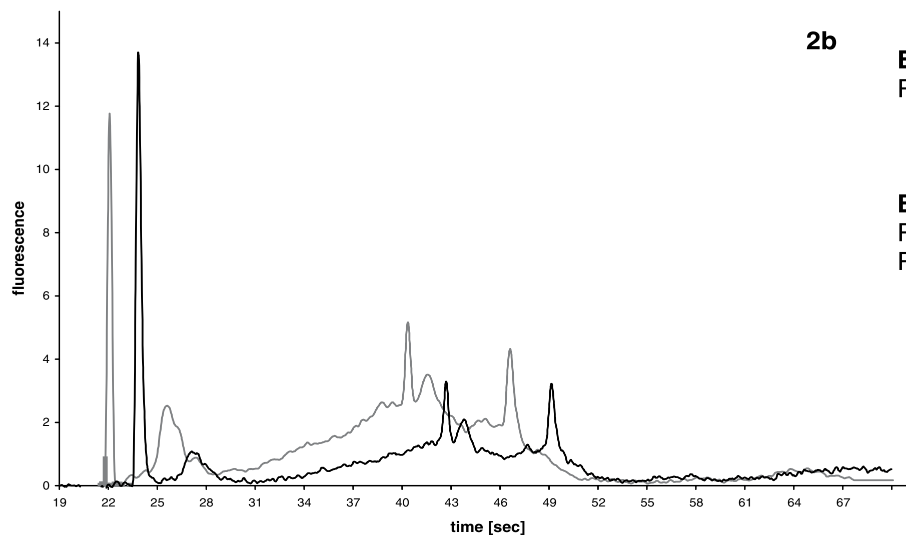


Fig. 1. Comparison of the 28S/18S ribosomal RNA ratios in Experion (solid line) and 2100 Bioanalyzer (dotted line). (A) 200 ng/µl input concentration (n = 36); (B) 50 ng/µl input concentration (n = 36)





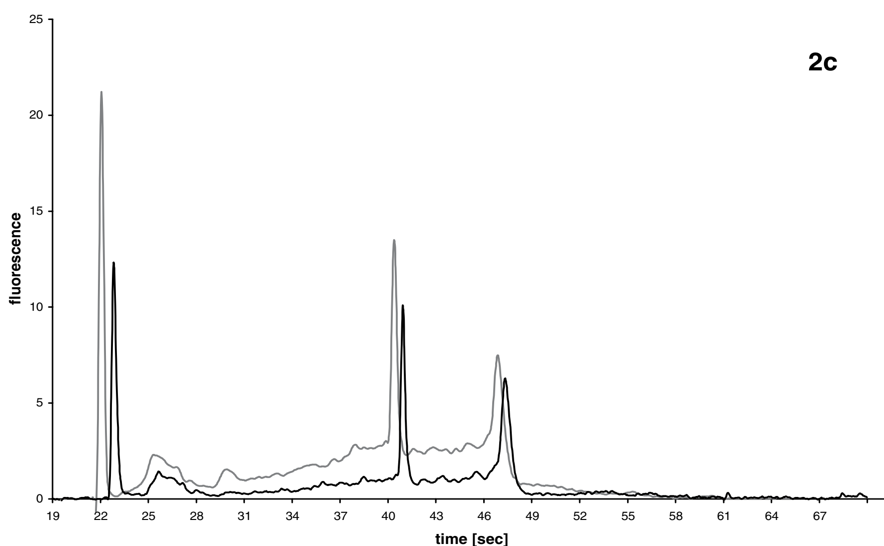


Fig. 2a – 2c. Comparisons of identical total RNA samples (50 ng/µl) which were assessed in both separation systems, in Experion (dotted line) and 2100 Bioanalyzer (solid line). Total RNA was extracted from bovine corpus luteum (**2a - 2b**) and bovine caecum (**2c**)

TABLE 1

Comparison of the gained area results

System	Experion Ladder	2100 Bioanalyzer Ladder
Experion (mean area units)	400.66	496.92
Deviation	96.28	40.35
CV [%]	24.0	8.1
2100 Bioanalyzer (mean area units)	220.93	218.8
Deviation	18.78	52.29
CV [%]	8.5	24.2

The 28S/18S rRNA ratios increased with the rise of RNA quality more significant in the 2100 Bioanalyzer compared to the Experion (figure 1). The detailed analysis on both separation systems reveals that ribosomal 28S/18S ratio inadequately describes RNA integrity.

Measured total RNA concentration

In addition analysis for the concentration values was done on 724 RNA profiles with the low concentrated samples (50 ng/µl and well), where 31 profiles were not obtained due to device problems during the runs (4.3%). The system automatically calculated the RNA concentration, according to the initially standard curve. The distributions of the computed concentration values were noticeably different between both separation systems. The Experion quantification showed higher accuracy (figure 3), significant higher values ($p<0.001$), and more reproducible mean concentrations: 54.2 ng/µl (CV=39.1%), compared to the 2100 Bioanalyzer 43.4 ng/µl (CV=57.1%). Using 200 ng/µl total RNA per run, the analysis was performed on 80 RNA profiles in the Experion and 91 RNA profiles in the 2100 Bioanalyzer, where 8 samples failed (4.7%). The distribution of the computed concentration values were significantly different ($p=0.025$): mean 211.1 ng/µl (CV=14.7%) for Experion and 235.8 ng/µl (CV=27.4%) for the 2100 Bioanalyzer (figure 3).

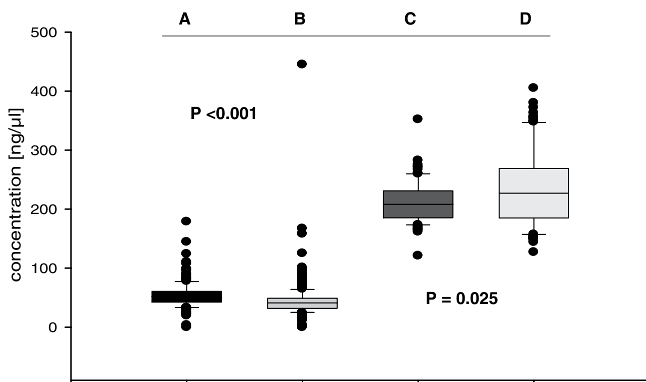


Fig. 3. Calculated concentration by both separation systems plotted in a box plot. (**A**) Experion 50 ng/µl (n = 198); (**B**) Bioanalyzer 50 ng/µl (n = 526); (**C**) Experion 200 ng/µl (n = 80); (**D**) Bioanalyzer 200 ng/µl (n = 91)

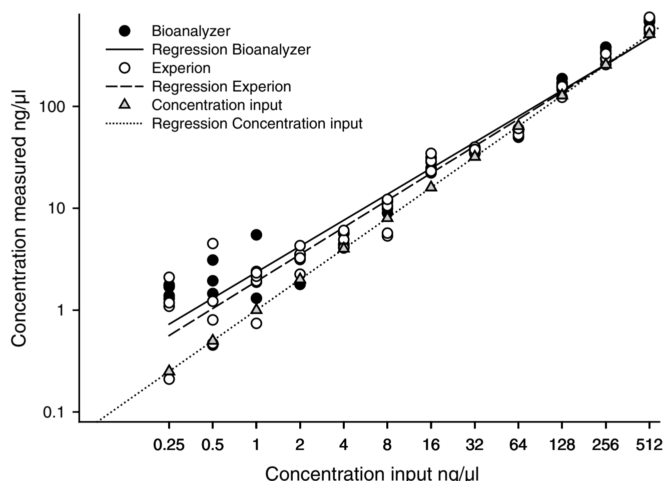


Fig. 4. Total RNA concentration measured by the Experion (dashed line) and the 2100 Bioanalyzer (solid line) versus total RNA input

Sensitivity of both separation systems

For testing the sensitivity a total RNA extract of bovine intestine was diluted to different concentration levels and then analyzed by both systems in parallel (n = 48). The concentrations per run were 512 ng/µl, 256 ng/µl, 128 ng/µl, 64 ng/µl, 32 ng/µl, 16 ng/µl, 8 ng/µl, 4 ng/µl, 2 ng/µl, 1 ng/µl, 0.5 ng/µl, 0.25 ng/µl. Regarding concentration measurements it can be observed that both platforms have high sensitivity down to 250 pg total RNA per run (figure 4), with high significant linearity ($p<0.001$) as shown by the linear regressions:

Experion: measured concentration = -1.404 + (1.190* input concentration); $r^2 = 0.953$

Bioanalyzer: measured concentration = -0.379 + (1.297* input concentration); $r^2 = 0.955$

But both systems overstate the concentrations determined in the BioPhotometer (Eppendorf) and the NanoDrop 1000 (Peqlab). Regression equantion show that the measurements done by the Experion are more close to the real input concentrations than those of the 2100 Bioanalyzer. The median overestimation of the Experion is 19.0% and that of the 2100 Bioanalyzer 29.7%, retrieved from the slope of the regression line.

Sensitivity of the 28S/18S rRNA measurement

Further the sensitivity of the 28S/18S rRNA measurement was determined in a serial dilution row as described above (512 ng/µl – 0.25 ng/µl, n=48). The Experion showed a decrease 28S/18S rRNA ratio correlating to lower RNA concentrations (**Fig. 5**). The fact that the 2100 Bioanalyzer graph shows an abrupt decrease corresponding to the concentrations lower than 8 ng/µl, which might be due its lower sensitivity. At concentrations lower than 1 ng/µl the 2100 Bioanalyzer does not measure any 28S/18S rRNA ratio. In both systems quantitation at higher concentrations is more exact what is visualized by the smaller error bars (**Fig. 5**).

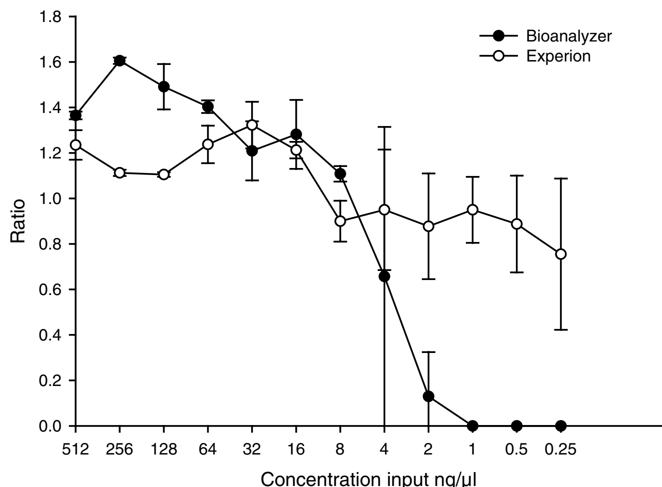


Fig. 5. 28S/18S ribosomal RNA Ratio versus total RNA input concentration

In regard to RNA quantitation and 28S/18S ratios, more accurate measurements are achieved by the Experion, especially at low concentration levels. The greater detection sensitivity of the Experion system allows the generation of a higher signal-to-noise ratio. In consequence the consumption of smaller amounts of RNA sample per measurement is possible.

Validity of the RNA ladder run

The ladder electropherogram of the Experion and the 2100 differ from each other concerning the number and sizes of the contained fragments. The Experion ladder electropherogram shows nine peaks, whereas the 2100 Bioanalyzer ladder displays only seven. Both ladders were pipetted in both separation systems; in the ladder well and in the fist sample well (figure 6). The ladder appearance and the ladder quality were evaluated in 37 runs on each platform. 62.2% of all ladder runs and 75.7% of all sample runs made with the Experion can be declared as good. In contrast, 86.5% and 88.3% of both the ladder and the sample runs provided by the 2100 Bioanalyzer are defined as successful runs. Higher validity and lower fluctuation of the ladder run is given by the 2100 Bioanalyzer system.

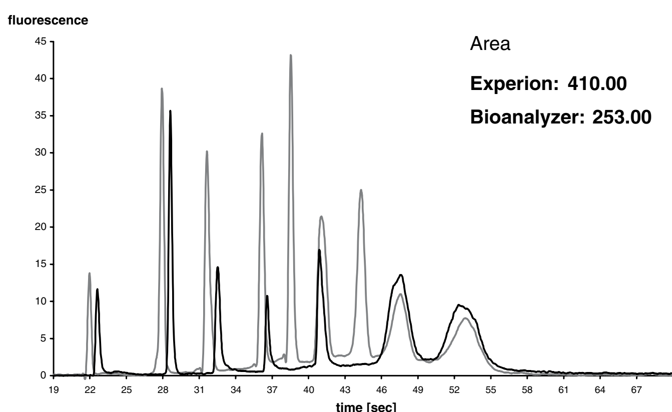


Fig. 6. Electropherogram of both ladders. Experion (dotted line) and 2100 Bioanalyzer (solid line)

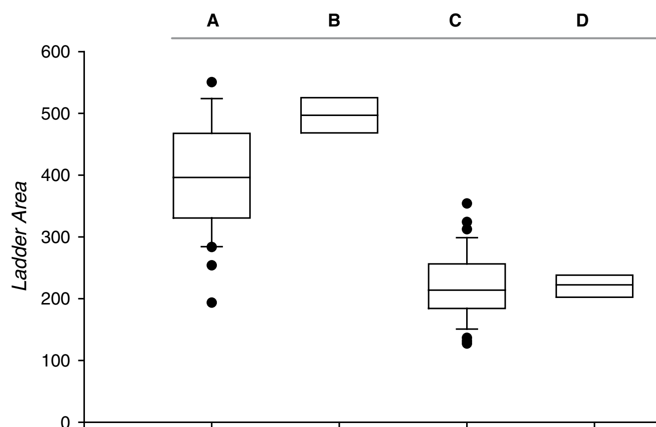


Fig. 7. Comparison of the ladder area on both capillary electrophoresis systems. (**A**) Experion ladder area (n = 37); (**B**) 2100 Bioanalyzer ladder performed in Experion (n = 4); (**C**) Bioanalyzer ladder area (n = 37); (**D**) Experion ladder run performed in 2100 Bioanalyzer (n = 4)

The Experion showed an average ladder area about 400.6 area units with a variance of 24.0%. The 2100 Bioanalyzer showed a variance of 24.2% and a smaller average ladder area unit about 218.8 (table 1; figure 7). Therefore the reproducibility of the ladder area for both systems is equivalent. Agilent Technologies defines a ladder area around 200 area units as normal; this corresponds to a concentration of 150 ng RNA. In contrast, Bio-Rad does not have a definition of a ladder area. Measuring the ladders vice versa on the both platforms, the Experion Ladder performed in the 2100 Bioanalyzer show a CV of ~8.5% and the Experion show a CV of ~ 8.1% for analyzing the 2100 Bioanalyzer ladder. The low statistical spread in C and D can be explained by considering that only a limited number of samples were used (figure 6).

Comparison of the Runs

Higher validity and lower fluctuation of the ladder run was given by the 2100 Bioanalyzer system. The Experion ladder area is roughly two-fold of the internal area units, therefore the 2100 Bioanalyzer and so the standard deviation as expected if the two systems were fully comparable. This indicates that the reproducibility of the ladder areas for the two systems is equivalent, regardless whether or not the value is stated.

Conclusions

The Experion system showed greater sensitivity of detection and provides a better quantity assessment of RNA samples. The RNA concentration measurements are less accurate and less reproducible, as they are most similar to the UV-Spectrophometer or the NanoDrop 1000. The reproducibility of both systems is nearly identical when used for RNA quantitation. The distribution of data illustrates that the chip-to-chip variations in both accuracy and reproducibility were very comparable. One advantage of the 2100 Bioanalyzer is that the system offers the opportunity of two quality measurements; the 28S/18S ribosomal RNA ratio and the RNA integrity number (RIN), whereas the Experion only offers the ribosomal ratio. It was revealed, that the ribosomal 28S/18S ratio is inadequately

to describe RNA integrity. A general recommendation couldn't be expressed, because both systems offer same applications and good handling. The Experion system is more convenient through the automatic priming station, which might be the reason of more reproducibility and higher sensitivity in the lower RNA range.

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Appendix II

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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.

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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.

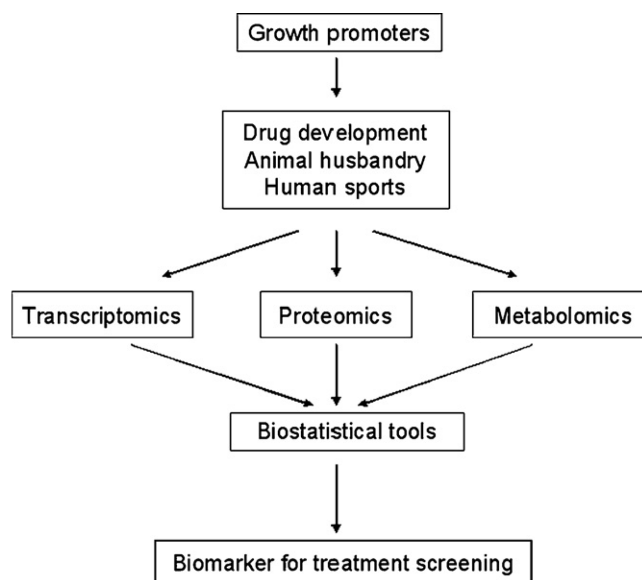

Fig. 1. Scheme of the use of *omic* technologies to trace anabolic hormone functions.

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

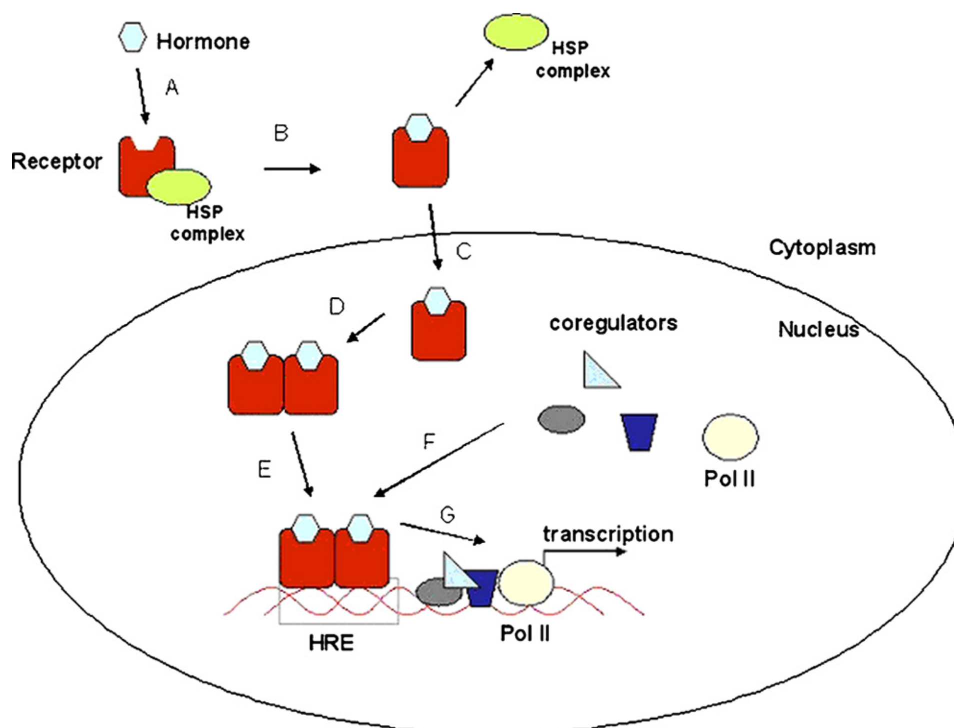

Fig. 2. Schematic diagram of the activation of a cytoplasmic steroid hormone receptor. After hormone binding (A) the HSP complex dissociates from the receptor (B), the hormone receptor complex translocates to the nucleus (C), dimerizes (D) and binds to a hormone responsive element (HRE) in the promoter 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.

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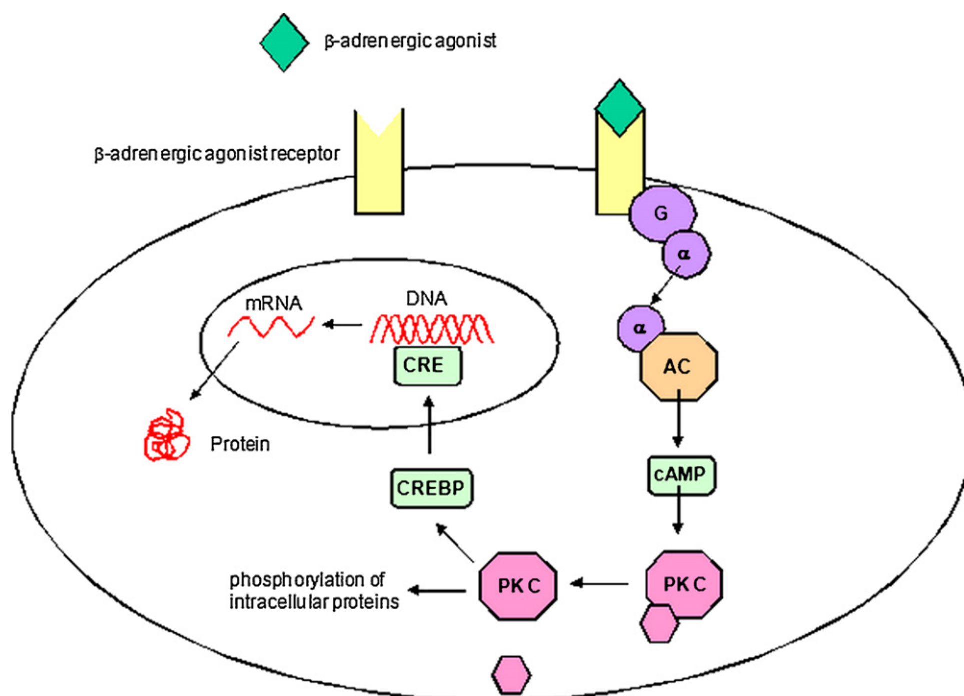

Fig. 3. Signaling cascade leading to the physiological mechanisms of β-adrenergic agonists.

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.

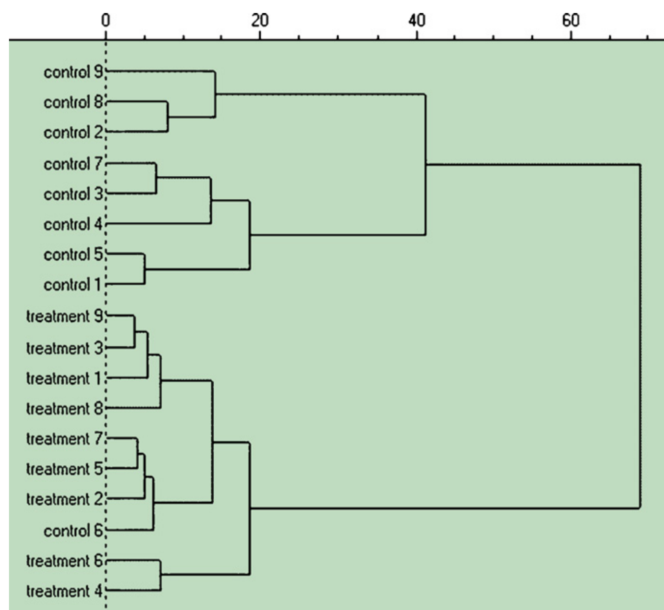

Fig. 4. Cluster dendogram of a qRT-PCR experiment with hormone treated and untreated heifers. Quantitative RT-PCR expression results of vaginal epithelial cells of untreated (control) and treated (treatment) heifers were clustered using GenEx version 4.3.6 Software (MultiD Analyses AB, Gothenburg, Sweden).

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

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

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Appendix III

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Influence of testosterone and a novel SARM on gene expression in whole blood of *Macaca fascicularis* ☆

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ABSTRACT

Anabolic hormones, including testosterone, have been suggested as a therapy for aging-related conditions, such as osteoporosis and sarcopenia. These therapies are sometimes associated with severe androgenic side effects. A promising alternative to testosterone replacement therapy are selective androgen receptor modulators (SARMs). SARMs have the potential to mimic the desirable central and peripheral androgenic anabolic effects of testosterone without having its side effects.

In this study we evaluated the effects of LGD2941, in comparison to testosterone, on mRNA expression of selected target genes in whole blood in an non-human model. The regulated genes can act as potential blood biomarker candidates in future studies with AR ligands.

Cynomolgus monkeys (*Macaca fascicularis*) were treated either with testosterone or LGD2941 for 90 days in order to compare their effects on mRNA expression in blood. Blood samples were taken before SARM application, on day 16 and on day 90 of treatment.

Gene expression of 37 candidate genes was measured using quantitative real-time RT-PCR (qRT-PCR) technology.

Our study shows that both testosterone and LGD2941 influence mRNA expression of 6 selected genes out of 37 in whole blood. The apoptosis regulators CD30L, Fas, TNFR1 and TNFR2 and the interleukins IL-12B and IL-15 showed significant changes in gene expression between control and the treatment groups and represent potential biomarkers for androgen receptor ligands in whole blood.
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1. Introduction

Over the last decades the proportion of elderly people in the population has increased [1]. This is the reason why the incidence of age-related conditions like sarcopenia and osteoporosis is rising and becoming one of the major topics in health care. Sarcopenia is the loss of muscle mass during the aging process that may lead to frailty [2–5]. Sarcopenia is commonly associated with osteoporosis, which is the age-related loss of bone mineral density. The combination of sarcopenia and osteoporosis results in a high incidence of bone fractures relating to accidental falls, which is a significant cause of morbidity and mortality in the elderly population.

Both conditions are associated with a decrease in the endogen production of anabolic hormones, including testosterone [4]. Testosterone treatment has been proposed as a therapy for osteoporosis and frailty in both men and women [6,7]. However, the androgen therapies available today are associated with androgenic side effects, such as skin virilization in women and prostate hypertrophy in men [8–10].

A promising alternative for testosterone replacement therapy is the development of selective androgen receptor modulators (SARMs) [6]. SARMs are synthetic molecules that bind to the androgen receptor exhibiting tissue-selective effects. An "ideal" SARM is an orally active compound that provides an increase in muscle mass and strength and has an anabolic effect on bone density without inducing undesirable androgenic side effects [6]. LGD2941 is a novel non-steroidal, orally active SARM, which has shown potent anabolic activity on bone and muscle in rats and monkeys, but reduced effects on the prostate [7].

It is already known that androgens cause changes in the biochemical pathways of different organs and tissues. Specific enzymes, receptors and cytokines can be activated or suppressed on the cellular mRNA expression level. Using appropriate specific and sensitive quantification methods, like quantitative real-time RT-PCR, such mRNA expression changes are measurable.

The aim of this study was to evaluate the effects of LGD2941, in comparison to testosterone, on mRNA expression of selected target genes in whole blood samples. Whole blood is chosen because samples can easily been taken from the living organism. Furthermore there is evidence in the literature that androgens affect gene

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expression of the different blood cells. The regulated genes have the potential to act as blood biomarkers in future studies with AR ligands.

2. Materials and methods

2.1. Animal experiment

24 male cynomolgus monkeys (*Macaca fascicularis*) were separated to four groups of six animals each. All animals were 5–6 years old, skeletally mature and had an average body weight of 6± kg. The treatments were group 1 (control or oral vehicle group), group 2 (reference group, testosterone group) 3.0 mg/kg Testosteronenanthate as Testoviron®-depot-250 (Schering, Berlin, Germany), dosed biweekly by intramuscular injection, group 3 (intermediate concentration group, SARM1) 1 mg/kg SARM LGD2941 daily and group 4 (high concentration group, SARM10) 10 mg/kg SARM LGD2941 daily. The oral vehicle control and the SARM were dosed once daily for 90 days.

Whole blood samples were taken at three time points. Predose samples were taken after study start without prior treatment. Further samples were taken at day 16 and day 90 of treatment. Duplicate blood samples (2.5 mL each) were transferred into PAXgene blood RNA tubes (BD, Heidelberg, Germany) gently shaken, incubated at room temperature for two hours and stored at −20 °C.

The animal attendance and blood sampling were done by Covance Laboratories GmbH (Münster, Germany) and was conducted with permission from the local veterinary authorities and in accordance with accepted standards of Humane Animal Care.

2.2. RNA preparation and qRT-PCR

RNA from blood samples was extracted using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

To quantify the amount of total RNA extracted, optical density (OD) was measured with the Biophotometer (Eppendorf Biophotometer, Hamburg, Germany) for each sample. RNA purity was calculated with the $OD_{260/280}$ ratio.

RNA integrity and quality control was performed via capillary electrophoresis in the Bioanalyzer 2100 (Agilent Technology, Palo Alto, USA). Eukaryotic total RNA Nano Assay (Agilent Technology) was taken for sample analysis and the RNA Integrity Number (RIN) served as RNA quality parameter. Agilent Bioanalyzer 2100 calculated the RIN value based on a numbering system from 1 to 10 (1 being the most degraded profile, 10 being the most intact) for all samples. A RIN ≥ 6 should be achieved to assure good results in qRT-PCR [11,12].

Candidate genes were chosen by screening the respective literature for androgen and inflammation-related effects on blood cells. Their expression was investigated using listed primers (Table 1). All primers were designed using published human nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Primer design and optimization was done with primer design program of MWG Biotech (MWG, Ebersberg, Germany) and primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with regard to primer dimer formation, self-priming formation and a constant primer annealing temperature of 60 °C. Newly designed primers were ordered and synthesized at MWG Biotech (Ebersberg, Germany) or Invitrogen (Karlsruhe, Germany). Primer performance testing was done with six optional untreated samples and a no template control (NTC contains only RNAse free water) for each primer set.

Quantitative real-time RT-PCR was performed using SuperScript III Platinum SYBR Green One-Step qPCR Kit (Invitrogen, Carlsbad, USA) by a standard protocol, recommended by the manufacturer. With the kit the master mix was prepared as follows: for one sample it is 5 μL 2× SYBR Green Reaction Mix, 0.5 μL forward primer (10 pmol/μL), 0.5 μL reverse primer (10 pmol/μL) and 0.2 μL SYBR Green One-Step Enzyme Mix (Invitrogen, Carlsbad, USA). 6.2 μL of the master mix was filled in the special 100 μL tubes and 3.8 μL RNA (concentration 1 ng/μL respectively 10 ng/μL) was added. Tubes were closed, placed into the Rotor-Gene 3000 and Analysis Software v6.0 was started (Corbett Life Science, Sydney, Australia). The following one-step qRT-PCR temperature cycling program was used for all genes: Reverse transcription took place at 55 °C for 10 min. After 5 min of denaturation at 95 °C, 40 cycles of real-time PCR with 3-segment amplification were performed consisting of 15 s at 95 °C for denaturation, 30 s at primer dependent temperature for annealing and 20 s at 68 °C for polymerase elongation. The melting step was then performed with slow heating starting at 60 °C with a rate of 0.5 °C per second up to 95 °C with continuous measurement of fluorescence.

Take off points (Ct) and melting curves were acquired by using the "*Comparative quantitation*" and "*Melting curve*" program of the Rotor-Gene 3000 Analysis software v6.0. Only genes with melting curves showing a single peak and no primer dimers were taken for further data analysis. Samples that showed irregular melting peaks were excluded from the quantification procedure.

2.3. Selection of target genes

Candidate genes that might be biomarkers in blood were chosen by screening the respective literature for androgen and inflammation-related effects on blood cells. Androgens are known to down-regulate proliferation of lymphocytes [13,14]. Therefore the different pro- and anti-inflammatory interleukins (IL) IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12B, IL-13 and IL-15 and the growth factors tumor growth factor β (TGF-β), insulin growth factor 1 receptor (IGF-1R) were selected for analysis. It was already shown that testosterone influences the rate of apoptotic blood cells [15–17]. Therefore different apoptosis regulators were chosen for analysis: the TNF receptor superfamily member 6 (Fas), its ligand FasL, tumor necrosis factor receptor (TNFR) 1 and 2, their ligand tumor necrosis factor α (TNF-α), B-cell CLL/lymphoma 2 (BCL-2), BCL2-like 1 (BCL-XL), Caspase 3 (Casp 3), Caspase 8 (Casp 8), CD30 Ligand (CD30L), the inflammatory factor nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFκB) and its inhibitor IκB. To determine if the treatment also has an influence on the amount of the different white blood cells, the expression of the cell specific CD Antigens CD4 (T helper cells), CD8 (cytotoxic T cells), CD11b (granulocytes), CD14 (monocytes), CD20 (B-cells), CD25 (activated T cells) and CD69 were measured. Further leukocyte genes that were measured are androgen receptor (AR), tumor necrosis factor β (TNF-β) and CD27 Ligand (CD27L). As genes expressed in reticulocytes, haemoglobin alpha (α-globin), haemoglobin beta (β-globin) and their transcription factors and stabilization factors transcription factor CP2 (CP2), acid phosphatase 1 (αCP1) and upstream transcription factor 1 (USF-1) were chosen. As reference gene candidates β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured, whereas β-Actin and GAPDH were chosen as best reference genes by using GenEx Ver 4.3.3 Software (multiD Analyses AB, Gothenburg, Sweden).

2.4. Data analysis and statistics

Statistical description of the expression data as well as statistical tests were produced with SAS v. 9.1.3 for Windows. The raw data were the Ct values obtained from each qPCR sample. Each qRT-PCR sample was associated with a blood sample whereas for each experimental animal two blood samples were analysed. Since the

Table 1
List of primer pairs used for qRT-PCR analysis.

Group	Gene	Primer name	Primer sequence 5′ → 3′	Product length
Reference genes	Ubiquitin C	UBC_for	TGA AGA CTC TGA CTG GTA AGA CC	128 bp
		UBC_rev	CAT CCA GCA AAG ATC AGC CTC	
	Actin-β	ActB_for	AGT CCT GTG GCA TCC ACG AA	148 bp
		ActB_rev	GCA GTG ATC TCC TTC TGC ATC	
	GAPDH	GAPDH_for	GAA GGT GAA GGT CGG AGT CAA	233 bp
		GAPDH_rev	GCT CCT GGA AGA TGG TGA TG	
Interleukins	IL-1β	IL1beta_for	GGA CAG GAT ATG GAG CAA CAA G	121 bp
		IL1beta_rev	AAC ACG CAG GAC AGG TAC AG	
	IL-2	IL2_for2	GCA ACT CCT GTC TTG CAT TGC	165 bp
		IL2_rev2	CAT CCT GGT GAG TTT GGG ATT C	
	IL-4	IL4_for3	TTC CCC CTC TGT TCT TCC TG	121 bp
		IL4_rev3	GTT GTG TTC TTC TGC TCT GTG AG	
	IL-6	IL6_for5	AGG AGA CTT GCC TGG TGA AA	179 bp
		IL6_rev5	CAG GGG TGG TTA TTG CAT CT	
	IL-10	IL10_for2	AGC CTTCGTC TGA GAT GAT CCA G	190 bp
		IL10_rev2	CAT TCT TCA CCT GCT CCA CG	
	IL-12b (p40)	IL12B_for	AAG GAG GCG AGG TTC TAA GC	213 bp
		IL12B_rev	AAG AGC CTC TGC TGC TTT TGA C	
	IL-13	IL-13_for2	AAT GGC AGC ATG GTA TGG AGC	124 bp
		IL-13_rev2	AGA ATC CGC TCA GCA TCC TC	
	IL-15	IL-15_for2	TCC AGT GCT ACT TGT GTT TAC TTC	93 bp
		IL-15_rev2	TAG GAA GCC CTG CAC TGA AAC	
Apoptosis regulators	Fas	FasR_for4	TTC TGC CAT AAG CCC TGT CC	174 bp
		FasR_rev4	CCA CTT CTA AGC CAT GTC CTT C	
	Fas ligand	FasL_for2	GGC CTG TGT CTC CTT GTC AT	162 bp
		FasL_rev2	GTG GCC TAT TTG CTT CTC CAA AG	
	TNFR1	TNFR1_for	AGC TGC TCC AAA TGC CGA AAG	147 bp
		TNFR1_rev	CAG AGG CTG CAA TTG AAG CAC	
	TNFR2	TNFR2_for	TGA CCA GAC AGC TCA GAT GTG	99 bp
		TNFR2_rev	TCC TCA CAG GAG TCA CAC AC	
	bcl-2	bcl2_for2	GAG GAT TGT GGC CTT CTT TGA G	170 bp
		bcl2_rev2	ACA GTT CCA CAA AGG CAT CCC	
	TNF-α	TNFa_for	AGG GAC CTC TCT CTA ATC AGC	104 bp
		TNFa_rev	CTC AGC TTG AGG GTT TGC TAC	
	Caspase 3	Casp3_for	GAA TTG ATG CGT GAT GTT TC	198 bp
		Casp3_rev	GCA GGC CTG AAT AAT GAA AAG	
	Caspase 8	Casp8_for	TGG CAC TGA TGG ACA GGA G	230 bp
		Casp8_rev	GCA GAA AGT CAG CCT CAT CC	
	bcl-xl	bcl-xl_for	TAA ACT GGG GTC GCA TTG TG	145 bp
		bcl-xl_rev	TGG ATC CAA GGC TCT AGG TG	
	CD30L	CD30L_for	CAT TCC CAA CTC ACC TGA CAA C	281 bp
		CD30L_rev	GCT CCA ACT TCA GAT CGA CAG	
Growth factors	TGF-β	TGFb_for	TAC TAC GCC AAG GAG GTC AC	239 bp
		TGFb_rev	AGG TAT CGC CAG GAA TTG TTG C	
	IGF-1R	IGF1R_for	CAT TTC ACC TCC ACC ACC AC	151 bp
		IGF1R_rev	AGG CAT CCT GCC CAT CAT AC	
CD antigens	CD4	CD4_for3	CTA AGC TCC AGA TGG GCA AG	154 bp
		CD4_rev3	TGA GTG GCT CTC ATC ACC AC	
	CD8	CD8_for	GGA CTT CGC CTG TGA TAT CTA C	112 bp
		CD8_rev	AAA CAC GTC TTC GGT TCC TGT G	
	CD11b	CD11b_for	GAG AAC AAC ATG CCC AGA ACC	246 bp
		CD11b_rev	CGG TCC CAT ATG ACA GTC TG	
	CD14	CD14_for	AGA ACC TTG TGA GCT GGA CG	115 bp
		CD14_rev	ATG GAT CTC CAC CTC TAC TGC	
	CD20	CD20_for	CAA CTG TGA ACC AGC TAA TCC C	163 bp
		CD20_rev	CCA TTC ATT CTC AAC GAT GCC AG	
	CD25	CD25_for	ATC AGT GCG TCC AGG GAT AC	196 bp
		CD25_rev	ACG AGG CAG GAA GTC TCA C	
	CD69	CD69_for2	TTG GCT ACC AGA GGA AAT GCC	164 bp
		CD69_rev2	CAG TCC AAC CCA GTG TTC CT	
Transcription factors	NFκB	NFkB_for2	ATC ATC CAC CTT CAT TCT CAA CTT G	149 bp
		NFkB_rev2	ATC CTC CAC CAC ATC TTC CTG	
	IκB α	IkappaB_for	AAC AGG AGG TGA TCG ATA AGC TG	138 bp
		IkappaB_rev	CCT TGT AGA TAT CCG CCT GG	
Reticulocyte genes	α-globin	alpha-gl._for	AGA CCT ACT TCC CGC ACT TC	275 bp
		alpha-gl._rev	CAG AAG CCA GGA ACT TGT CC	
	β-globin	beta-gl._for	GTC CAC TCC TGA TGC TGT TAT G	240 bp
		beta-gl. rev	TGT CAC AGT GCA GCT ACA TC	
	αCP1	aCP1_for	CCA CCC ATG AAC TCA CCA TTC	160 bp
		aCP1_rev	GCA GAG CCA GTG ATA GTA ACC	
	USF1	USF1_for	AGA TTC AGG AAG GTG CAG TGG	121 bp
		USF1_rev	CCA TTC TCA GTT CGG AAG ACG	

Table 1 (*Continued*)

Group	Gene	Primer name	Primer sequence 5′ → 3′	Product length
	CP2	CP2_for3	TCT TCG TTT ACC ATG CCA TCT ATC	178 bp
		CP2_rev3	CAT GCT TCT TCC TGA AAG TTC TG	
Other genes	Androgen receptor	AR_for	CCA CTT CCT CCA AGG ACA ATT AC	126 bp
		AR_rev	TGG ACT CAG ATG CTC CAA CG	
	TNFβ	TNFb_for	TGC TCA CCT CAT TGG AGA CC	149 bp
		TNFb_rev	AGT AGA CGA AGT AGA TGC CAC TG	
	CD27L	CD27L_for	ACA GGA CCT CAG CAG GAC	272 bp
		CD27L_rev	GAG GCA ATG GTA CAA CCT TGG	

amplification efficiency was not known, the assumption of identical amplification efficiency 100% was made, allowing more simple quantification model.

The Ct values of each gene were averaged by arithmetic mean for each animal. The obtained mean Ct values were then translated to normalized expression quantities using two reference genes in a form of normalization index. The normalization index was calculated as an arithmetic mean of the Ct values of the two reference genes:

$$\text{reference index} = \text{mean}(\text{CtACTB}, \text{CtGAPDH}) \quad (1)$$

Then, the expression of every target gene was calculated relatively to the expression of the housekeeping gene as:

$$\text{normalized expression} = \frac{2^{\text{reference index}}}{2^{\text{Ct target gene}}}, \quad (2)$$

where the 2 represents the 100% amplification efficiency. The normalized expressions of the timepoints 16 and 90 days were then divided with the normalized expressions of the baseline (predose), generating the expression ratio R as:

$$R_{\text{timepoint/baseline}} = \frac{\text{normalized expression}_{\text{timepoint}}}{\text{normalized expression}_{\text{baseline}}} \quad (3)$$

The expression ratio R was then analysed statistically. The Box–Whisker plot was constructed to facilitate visual screening of regulated genes (Figs. 1–3).

The objective of the statistical analysis was to disclose genes with significant regulation between control group and any of the treated groups. It was not intended to perform all treatment-to-treatment tests for all genes in order to avoid statistical type I error (false positive difference). Hence, ANOVA model was calculated on the $\log_2$ transformed R values employing the SAS procedure GLM with contrast sentence defining the control group as the contrast group for all treatment groups, thus adjusting the overall test confidence level to the number of relevant comparisons only. Further adjustment of the overall confidence level with respect to number of investigated genes was not performed. Hence, this study is to be considered as purely explorative whereas significant findings here indicate candidate biomarkers. Tests generating significant ($p < 0.05$) results were reviewed based on descriptive parameters of the compared groups and visually by means of the Box–Whisker plots to disclose possible outliers. As comparable trends were observed between the three treatment groups, no further test were produced.

To disclose multivariate response to the treatment, the method of principal component analysis (PCA) was employed using GenEx v. 4.3.3 (multiD Analyses AB, Göteborg, Sweden). 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. Normalized expression values of all responding genes were taken as the initial variables and reduced to two principal components only, facilitating thus resolution of treatment clusters in the scatter plot (Fig. 4). Similarly, also each gene was analyzed by PCA taking its response in each sample as the initial variable and plotted in two dimensional scatter plot. This facilitated resolution of co-regulated genes (Fig. 5).

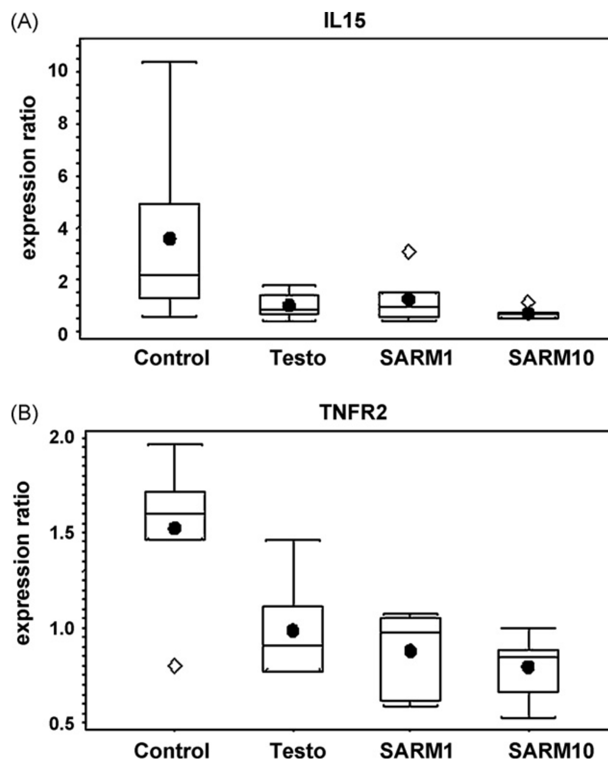

Fig. 1. Significant regulation for IL-15 (A) and TNFR2 (B) between control and treated samples after 16 days of treatment. Box plots show the median, mean (spot) and standard deviations.

3. Results

3.1. RNA quality

The mean (±std.dev.) RIN value of the blood samples were 7.5 (±4.8) at predose, 8.5 (±5.0) on day 16 and 7.7 (±4.2) at day 90 indicating a well intact RNA.

3.2. Primer testing and gel electrophoresis

Primer pairs of 40 genes were successfully used in qRT-PCR analysis to get single peaks and uniform melting curves, as well as a specific single band in high resolution agarose gel electrophoresis.

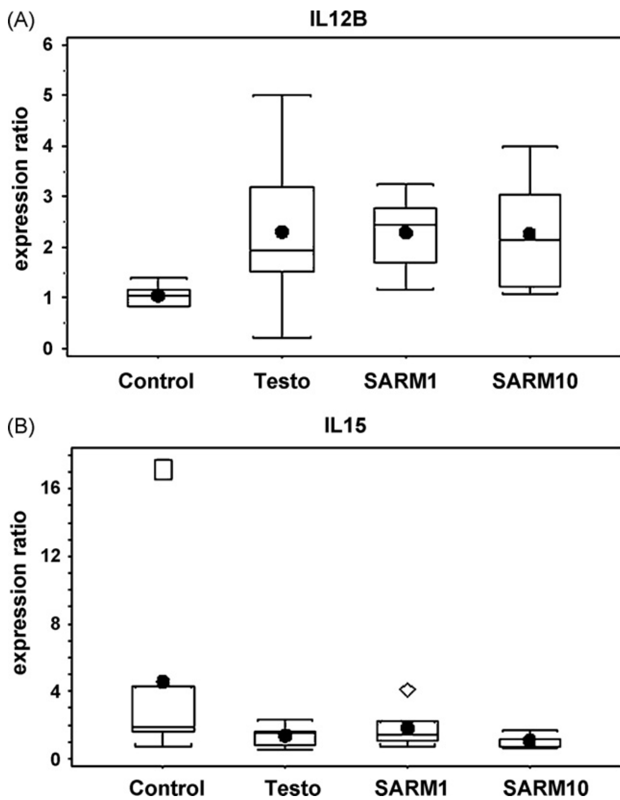

Fig. 2. Significant regulation for the proinflammatory interleukins IL-12B (A) and IL-15 (B), between control and treated samples after 90 days of treatment. Box plots show the median, mean (spot) and standard deviations.

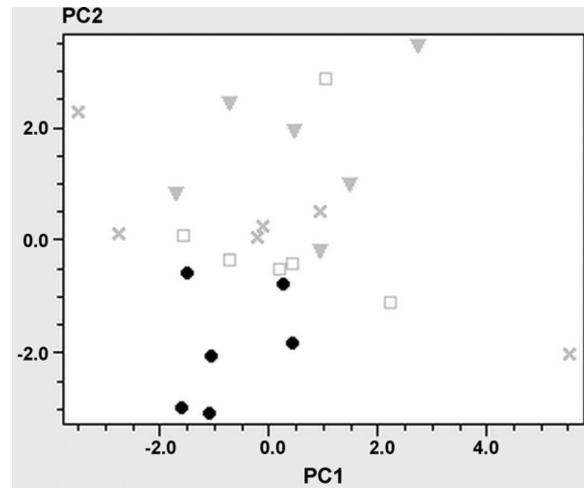

Fig. 4. Principal components analysis (PCA) for the six regulated genes IL-12B, IL-15, CD30L, Fas, TNFR1 and TNFR2 in the control group (black dots) the testosterone treated group (grey cross) the low dosed SARM group (grey squares) and the high dosed SARM group (grey triangle).

3.3. qRT-PCR results and data analysis

The calculation of the expression ratios (formula (1)) produced non-normally distributed data with frequent extreme values. Some of the extreme values can be outliers and were indicated in the Box–Whisker plot as squares outside the beyond inter quartile range (box). Nonetheless, no exclusion of extreme values/outliers was performed.

Significant down-regulation of gene expression of the treatment groups compared to the control group could be identified for IL-15 ($p = 0.0093$) and TNFR2 ($p < 0.0001$) after 16 days (Fig. 1)

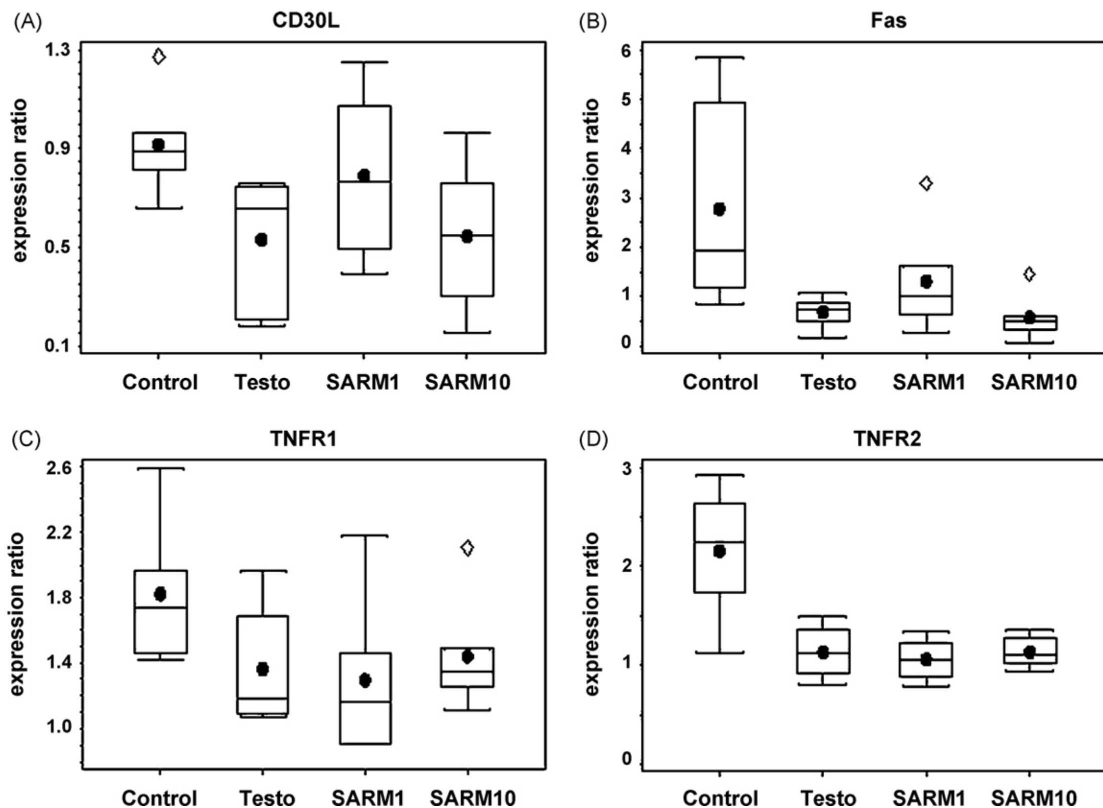

Fig. 3. Significant regulation for the apoptosis regulators CD30L (A), Fas (B), TNFR1 (C) and TNFR2 (D) between control and treated samples after 90 days of treatment. Box plots show the median, mean (spot) and standard deviations.

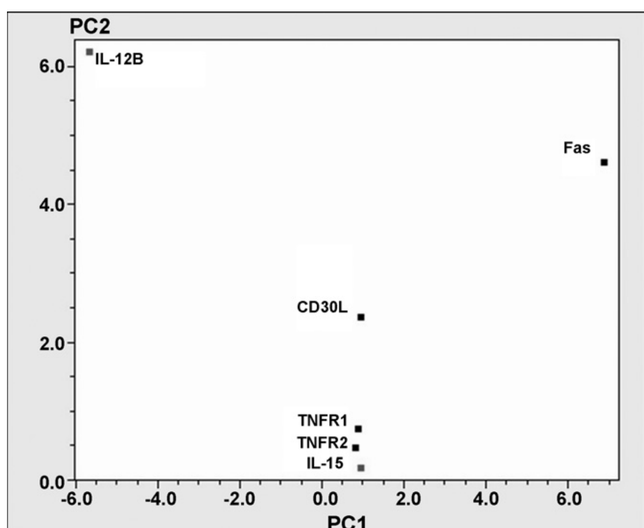

Fig. 5. Principle components analysis (PCA) for the regulated genes in all four groups. Grey spots show the interleukins and black spots show the apoptosis regulators.

and for IL-15 ($p = 0.0498$), CD30L ($p = 0.0435$), Fas ($p = 0.0032$), TNFR1 ($p = 0.0308$) and TNFR2 ($p < 0.0001$) after 90 days of treatment. Significant up-regulation of gene expression of the treatment groups compared to the control group could be observed for IL-12B ($p = 0.0240$) after 90 days of treatment (Figs. 2 and 3).

In the control group high variability could be observed compared to the treatment groups as indicated by the Box–Whisker plot. This reflects the natural variability of the non-induced expressing in each studied subject.

Principal components analysis (PCA) is a technique used to reduce multidimensional data sets to lower dimensions for analysis. Fig. 4 was obtained by plotting all samples of the four treatment groups by their two principal components obtained from the six responder genes. Black dots represent samples of the control group, grey crosses show the testosterone group, grey squares represent the SARM1 group and the grey triangles display the SARM10 group. A distinct control group can be seen, showing that there was a multi-transcriptional response to the treatment by any of the three drugs. In addition, the SARM1 neighbors to the control group, creating thus a transition to the Testosteron group and the SARM10 group. In Fig. 5 the six responder genes are clustered. Black dots show apoptosis regulators and grey spots display the interleukins. A distinct cluster of TNF receptors can be resolved.

4. Discussion

In this study changes of gene expression in blood cells caused by treatment with LGD2941 or testosterone were evaluated in order to compare the effects of both treatments on gene expression in blood cells. Further aims were the description of physiological effects and the identification of potential biomarkers for the treatment with AR ligands.

The main physiological effect that could be observed in this study is the down-regulation of various apoptotic marker genes in all three treatment groups. This is shown by the significant regulation ($p < 0.05$) of the apoptosis receptors Fas, TNFR1, TNFR2 and the apoptosis ligand CD30L. All regulated apoptosis factors belong either to the TNF Family (CD30L) or to the TNF-Receptor Family (TNFR1, TNFR2, Fas) [18]. It is already known that the death receptor Fas plays a dominant role in the programmed cell death of lymphocytes [18]. When B- and T-cells are activated they get sensitized to Fas mediated apoptosis. On resting peripheral lymphocytes Fas expression is low or even absent. Activation of B- and T-cells results in up-regulation of Fas mRNA [18–23]. Down-regulation of Fas after 90 days of treatment can be a hint to a down-regulating effect on the immune response. The death receptors TNFR1 and TNFR2 activate apoptosis via binding of TNF-α or TNF-β. Binding of the ligand to TNFR1 or TNFR2 can stimulate apoptosis and activate NFκB, whereas in most cases TNFR1 is responsible for these signals [18]. Ligand binding to TNFR2 leads to proliferation of thymocytes [24]. While TNFR2 expression is already regulated after 16 days of treatment, regulation of TNFR1 is only regulated after 90 days of treatment. A reason for this phenomenon could be that the mRNA expression of TNFR2 is inducible whereas expression of TNFR1 is not [24]. CD30L, a member of the TNF ligand superfamily is known to induce apoptosis by binding to its receptor CD30 and is expressed on activated T-cells [25,26]. Down-regulation of CD30L could also be observed after 90 days of treatment.

The down-regulation of these apoptosis regulators suggest that the immune response is suppressed by the treatment with testosterone and the SARM. This observation is consistent with the fact that testosterone has a suppressive effect on the immune system [27–29].

The gene expression of IL-12B – a subunit of IL12 – is up-regulated after 16 days of treatment. The main producers of IL-12 are monocytes, dendritic cells and activated macrophages. It promotes IFN-γ production by CD4 positive T-cells and stimulates proliferation and cytotoxic activity of T-cells and natural killer cells [30]. Gene expression of IL-15 is down-regulated after 16 and 90 days of treatment. It is produced by epithelial cells, fibroblasts, activated monocytes and dendritic cells. It acts as a T-cell activating factor but is not expressed by T-cells themselves [31]. Another important function of IL-15 is the up-regulation of natural killer cell survival and it promotes the production of IFN-γ, GM-CSF and TNF by natural killer cells [32–34].

Regarding the Box–Whisker plots it can be observed that the statistical variance in the control group is higher than in the treatment groups. The reason for this could be the natural variability of the non-induced expression in each studied subject. Suppression of gene expression by an external stimulus like treatment with testosterone or the SARM reduces natural variability of gene expression.

The PCA shows that both drugs show equivalent response and that the treatments differ from the control.

The second aim of this study was to find potential biomarkers for the use of the SARM. If the physiological effects of testosterone and the SARM are compared it became obvious that the SARM is active similar to natural androgens. The regulated genes found in this study can act as first biomarker candidates for the development of a screening pattern in whole blood. To confirm these biomarker candidate genes more studies will be helpful. In primary cell cultures or in further *in vivo* experiments it could be determined if the suggested parameters are independent of age, sex and immune status.

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Appendix IV

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Identification of potential gene expression biomarkers for the surveillance of anabolic agents in bovine blood cells

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ABSTRACT

In the EU, the use of anabolic steroids in food producing animals has been forbidden since 1988. The routine methods used in practice are based on the detection of hormonal residues. To overcome these routine methods, growth-promoting agents are sometimes administered at concentrations below the detection limit and new anabolic substances are designed. Therefore, new monitoring systems are needed to overcome the misuse of anabolic agents in meat production.

In this study, a new monitoring system was applied: the quantification of mRNA gene expression changes by quantitative real time reverse transcription polymerase chain reaction (qRT-PCR). Blood was selected as ideal tissue for biomarker screening. From the literature, it is known that steroid hormones affect mRNA gene expression of the different blood cells, which can easily be taken from the living animal.

In an animal trial, 18 Nguni heifers were separated to two groups of nine animals. One group served as untreated control and the other group was treated with a combination of trenbolone acetate plus estradiol for 39 days in order to allow the detection of the effect on mRNA expression in blood at three time points. Candidate genes used for developing a biomarker pattern were chosen by screening the actual literature for anabolic effects on blood cells.

It could be demonstrated that the combination of trenbolone acetate plus estradiol significantly influences mRNA expression of the steroid receptors (ER-α and GR-α), the apoptosis regulator Fas, the proinflammatory interleukins IL-1α, IL-1β and IL-6 and of MHCII, CK, MTPN, RBM5 and Actin-β. Advanced statistical analysis by Principal Components Analysis (PCA) indicated that these genes represent potential biomarkers for this hormone combination in whole blood.

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1. Introduction

Growth-promoting agents like anabolic steroids or β-agonists are used in meat producing animals to improve weight gain and feed efficiency in order to increase the productivity and to reduce production costs [13,20]. Due to adverse effects of hormone residues for the consumer [4,27] the use of anabolic hormones for growth promotion is forbidden in the European Union since 1988 under Directive 88/146/EEC. Routine methods like immuno assays, either radio immuno assay (RIA) or enzyme immuno assay (EIA), and chromatographical methods combined with mass spectrometry are used to detect hormone residues [18,19,26,29]. To avoid detection of residues during routine control, growth-promoting agents are often administered in cocktails with such low amounts per agent that residues are below the detection limit [1]. Alternatively, new compounds, not yet included in testing programs, are used. Therefore, it is necessary to develop new monitoring systems to detect a broad range of agents at the lowest concentration that is used to get a growth-promoting effect. A potential way to develop a new monitoring system is to find gene expression biomarkers for the illegal use of anabolic steroids [23,24,28].

It is well known that steroid hormones influence biochemical pathways of different organs and tissues. mRNA expression of hormone dependent genes can be activated or suppressed.

Using appropriate specific and sensitive quantification methods, like quantitative real time reverse transcription polymerase chain reaction (qRT-PCR), such mRNA expression changes are measurable at very low levels. From the literature it is known that sex steroid hormones show physiological effects on the different blood cells [3,9,16].

The aim of this pilot study was to monitor the effects of a commercially available combination of trenbolone acetate plus estradiol on mRNA expression of selected target genes in bovine whole blood and to perform a bioinformatic evaluation in order to find potential biomarkers for the effective surveillance of this hormone combination.

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2. Materials and methods

2.1. Animal experiment

18 healthy, nonpregnant, 2-year-old Nguni heifers were separated to two groups of nine animals each. One group was treated with Revalor H® (140 mg Trenbolone acetate plus 14 mg estradiol, Intervet, Isando, RSA) by implantation into the middle third of the pinna of the ear and one group was untreated serving as control.

Whole blood samples were taken at four time points. Predose samples were taken prior to treatment. Further samples were taken at day 2, day 16 and day 39 after treatment start. Blood samples (2.5 mL each) were transferred into PAXgene blood RNA tubes (BD, Heidelberg, Germany) gently shaken, incubated at room temperature for 2 h and stored at −20 °C. At the same time points a complete blood count was done by the section of clinical pathology, University of Pretoria, South Africa, to control the health status of the animals. The animal attendance and blood sampling were done by the Onderstepoort Veterinary Institute (Onderstepoort, Pretoria, South Africa). The animals were housed and fed according to practice.

2.2. Total RNA extraction and quality determination

Total RNA from blood samples was extracted using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

To quantify the amount of total RNA extracted, optical density (OD_{260}) was measured with the photometer (Eppendorf Biophotometer, Hamburg, Germany) for each sample. RNA purity was calculated with the $OD_{260/280}$ ratio.

RNA integrity and quality control was performed via capillary electrophoresis in the Bioanalyzer 2100 (Agilent Technology, Palo Alto, USA). Eukaryotic total RNA Nano Assay (Agilent Technology) was taken for sample analysis and the RNA Integrity Number (RIN) served as RNA quality parameter. Agilent Bioanalyzer 2100 calculated the RIN value based on a numbering system from 1 to 10 (1 being the most degraded profile, 10 being the most intact) for all samples.

2.3. RNA reverse transcription

Constant amounts of 1 μg total RNA were reverse transcribed to cDNA using the following master mix: 12 μL 5 × Buffer (Promega, Mannheim, Germany), 3 μL Random Hexamer Primers (50 mM; Invitrogen), 3 μL dNTP Mix (10 mM; Fermentas, St Leon-Rot, Germany) and 200 U of MMLV H-Reverse Transcriptase (Promega) according to the manufacturer's instructions.

2.4. Specific primer design

All primers were designed using published bovine nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Primer design and optimization was done with primer design program of MWG Biotech (MWG, Ebersberg, Germany) and primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with regard to primer dimer and self-priming formation. Newly designed primers were ordered and synthesized at MWG Biotech. Primer testing was performed with three optional samples and a no template control (NTC contains only RNAse free water). To determine the optimal annealing temperature for each primer set a temperature gradient PCR was done. All used primers are listed in Table 1 .

2.5. Quantitative PCR analysis

To analyze gene expression of candidate genes, qRT-PCR analysis was done using the iQ5 (Bio-Rad, Munich, Germany). Quantitative real-time RT-PCR was performed using MESA GREEN qPCR MasterMix Plus for SYBR® Assay w/fluorescein Kit (Eurogentec, Cologne, Germany) by a standard protocol, recommended by the manufacturer.

With the kit the master mix was prepared as follows: For one sample it is 7.5 μL MESA GREEN 2× PCR Master Mix, 1.5 μL forward primer (10 pmol μL^{-1}), 1.5 μL reverse primer (10 pmol μL^{-1}) and 3 μL RNase free water. For qPCR analysis 1.5 μL cDNA was added to 13.5 μL Master Mix. qPCR was performed in 96 Well Plates (Eppendorf, Hamburg, Germany) and pipetting was done by the epMotion 5075 (Eppendorf).

The following real-time PCR cycling protocol was employed for all investigated factors: denaturation for 5 min at 95 °C, 40 cycles of a two segmented amplification and quantification program (denaturation for 3 s at 95 °C, annealing for 10 s at primer specific annealing temperature listed in Table 1), a melting step by slow heating from 60 to 95 °C with a dwell time of 10 s and continuous fluorescence measurement. Threshold cycle (Ct) and melting curves were acquired by using the IQ5 Optical System software 2.0 (Bio-Rad). Only genes with clear melting curves were taken for further data analysis. Samples that showed irregular melting peaks were excluded from the quantification procedure.

2.6. Selection of candidate target genes

Candidate genes that might be biomarkers in blood were chosen by screening the respective literature for steroidal and inflammation related effects on blood cells.

It is known that steroid hormones affect the expression and mRNA stability of their receptors [10]. Therefore the steroid receptors androgen receptor (AR), estrogen receptor alpha (ER-α) and beta (ER-β) and the glucocorticoid receptor (GR-α) were chosen as candidate genes. It was already shown that testosterone influences the rate of apoptotic blood cells [3,9,16]. Different apoptosis regulators were included: TNF receptor superfamily member 6 (Fas), its ligand FasL, tumor necrosis factor receptor (TNFR) 1 and 2, their ligand tumor necrosis factor α (TNF-α), B-cell CLL/lymphoma 2 (BCL-2) and caspase 8 (Casp 8). Androgens are known to downregulate proliferation of lymphocytes [11,14]. Therefore a variety of pro- and anti-inflammatory interleukins (IL) (IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12B, IL-15) and the growth factors insulin-like growth factor 1 (IGF-1), tumor growth factor beta (TGF-β) and interferone gamma (IFN-γ) were analyzed. To determine if the treatment has as well an influence on the amount of the different white blood cells, the expression of the cell specific CD Antigens CD4 (T helper cells) and CD8 (cytotoxic T was measured. Further genes were the inflammatory factor nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFκB), major histocompatibility complex class II (MHC II), adrenergic beta kinase 2 (ADRBK2), actin-α 1 (ACTA1), creatin kinase (CK), jun oncogene (JUN), estrogen induced transcription factor (EITr), m yotropin (MTPN), tropomodulin 3 (TMOD3) and RNA binding protein 5 (RBM5). As reference gene candidates ubiquitin 3 (UB3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin-β (ACTB), histone and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) were measured whereas the UBC and GAPDH were chosen as best reference genes by using GenEx Ver 4.3.6 Software (MultiD Analyses AB, Gothenburg, Sweden).

2.7. Data analysis and statistics

Significant changes of the amount of the different blood cells between the two groups were determined using an unpaired t-test.

Table 1
List of primer pairs used for qRT-PCR analysis.

Gene group	Gen		Sequenz	Annealing temperature	Product length
Reference genes	GAPDH	for	GTC TTC ACT ACC ATG GAG AAG G	60 °C	197 bp
		rev	TCA TGG ATG ACC TTG GCC CAG		
	UB3	for	AGA TCC AGG ATA AGG GAA GGC AT	60 °C	198 bp
		rev	GCT CCA CCT CCA GGG TGA T		
Steroid receptors	AR	for	CCT GGT TTT TCA ATG AGT ACC GCA TG	62 °C	172 bp
		rev	TTG ATT TTT CAG CCC ATC CAC TGG A		
	ERalpha	for	AGG GAA GCT CCT ATT TGC TCC	60 °C	233 bp
		rev	GGT GGA TGT GGT CCT TCT C		
	ERb	for	TTA GCC ATC CAT TGC CAG CC	64 °C	248 bp
		rev	GCC TTA CAT CCT TCA CAC GAC		
	GRa	for	TTC GAA GAA AAA ACT GCC CAG C	64 °C	190 bp
		rev	CAG TGT TGG GGT GAG TTG TG		
Apoptosis regulators	FasL	for	CAT CTT TGG AGA AGC AAA TAG	60 °C	205 bp
		rev	GGA ATA CAC AAA ATA CAG CCC		
	Fas	for	TGT TGT CAG CCT TGT CCT CC	60 °C	174 bp
		rev	GTT CCA CTT CTA GCC CAT GTT C		
	bcl-2	for	ATG ACT TCT CTC GGC GCT AC	60 °C	245 bp
		rev	CCG GTT CAG GTA CTC GGT CA		
	TNFa	for	CCA CGT TGT AGC CGA CAT C	60 °C	155 bp
		rev	CCC TGA AGA GGA CCT GTG AG		
	TNFR1	for	TCC AGT CCT GTC TCC ATT CC	60 °C	236 bp
		rev	CTG GCT TCC CAC TTC TGA AC		
	Casp 8	for	TAG CAT AGC ACG GAA GCA GG	60 °C	294 bp
		rev	GCC AGT GAA GTA AGA GGT CAG		
	TNFR2	for	AGCAGCACGGACAAGAGG	60 °C	220 bp
		rev	CTGTGTCCCTCGTGGAG		
Interleukins	IL-1a	for	CCT CTC TCT CAA TCA GAA GTC C	64 °C	142 bp
		rev	CCA CCA TCA CCA CAT TCT CC		
	IL-1β	for	TTC TCT CCA GCC AAC CTT CAT T	60 °C	198 bp
		rev	ATC TGC AGC TGG ATG TTT CCA T		
	IL-6	for	GCT GAA TCT TCC AAA AAT GGA GG	60 °C	200 bp
		rev	GCT TCA GGA TCT GGA TCA GTG		
	IL-8	for	ATG ACT TCC AAG CTG GCT GTT G	60 °C	149 bp
		rev	TTG ATA AAT TTG GGG TGG AAA G		
	IL-10	for	TGA TGC CAC AGG CTG AGA ACC AC	64 °C	118 bp
		rev	TCG CAG GGC AGA AAG CGA TGA C		
	IL12B	for	TACACAGTGGAGTGTCAGGAG	60 °C	250 bp
		rev	TCAGGGAGAAGTAGGAATGCG		
	IL15	for	TTCCATCCAGTGCTACTTGTG	60 °C	127 bp
		rev	ACATACTGCCAGTTTGCTTCTG		
CD antigens	CD 4	for	TTC CTT CCC ACT CAC CTT CG	63 °C	132 bp
		rev	ATC TTG TTC ACC TTC ACC TCT C		
	CD 8	for	AGA AGG TGG AGC TGC AAT GCG AG	60 °C	294 bp
		rev	GCA AGA AGA CAG GCA CGA AGT TAC TGA AG		
Growth factors	IGF-1	for	CAT CCT CCT CGC ATC TCT TC	62 °C	238 bp
		rev	CTC CAG CCT CCT CAG ATC AC		
	TGFb	for	TTC ATG CCG TGA ATG GTG GCG	60 °C	167 bp
		rev	ACG TCA CTG GAG TTG TGC GG		
	IFNg	for	GCA GAT CCA GCG CAA AGC CAT AAA TG	60 °C	112 bp
		rev	TCT CCG GCC TCG AAA GAG ATT CTG AC		
Others	NFkB	for	GCC TGT CCT CTC TCA CCC CAT CTT TG	60 °C	149 bp
		rev	ACA CCT CGA TGT CCT CTT TCT GCA CC		
	YWHAZ	for	CAG GCT GAG CGA TAT GAT GAC	60 °C	141 bp
		rev	GAC CCT CCA AGA TGA CCT AC		
	ACTB	for	AAC TCC ATC ATG AAG TGT GAC	60 °C	202 bp
		rev	GAT CCA CAT CTG CTG GAA GG		
	Histon	for	ACTGCTACAAAAGCCGCTC	62 °C	233 bp
		rev	ACTTGCCTCCTGCAAAGCAC		
	ACTA1	for	TAT TGT GCT CGA CTC CGG C	63 °C	160 bp
		rev	GTC ACG AAG GAG TAG CCA C		
	CK	for	ATG ACA GAG CAG GAG CAG CA	60 °C	183 bp
		rev	ATG GAG ATG ACT CGG AGG TG		
	ADRBK2	for	ACC TAT GCC TTC CAC ACT CC	60 °C	121 bp
		rev	CGT AAA ACC GCA TCT CCT TC		
	MHC2	for	AAC CTA CAG TGA CCA TCT CCC	60 °C	108 bp
		rev	ACC ACC GAA CCT TGA TCT GG		
	JUN	for	ATCAAGGCAGAGAGGAAGCG	63 °C	217 bp
		rev	TTAGCATGAGTTGGCACCCG		
	EITr	for	GTTCCTCAATTCCGTCTTCATC	60 °C	216 bp
		rev	TCACTGTTCTCCTCTCATCTC		
	MTPN	for	ATT ATG CAG CAG ATT GTG GAC AG	60 °C	112 bp

Table 1 (*Continued*)

Gene group	Gen		Sequenz	Annealing temperature	Product length
		rev	TAGACGGCAGACAGAAGAGG		
	TMOD3	for	ATCTTGACCCTGAGAACGCC	64 °C	142 bp
		rev	TCTTCCCTGTCCTTATGCTCC		
	RBM5	for	CCA TCA CGG AGA GCG ATA TTC	60 °C	164 bp
		rev	TTTCTGATTGGCTTCCATCCAG		

Statistical description of the expression data as well as statistical tests were produced with Sigma Stat 3.0. The raw data were the Ct values obtained from each qPCR sample. Each qPCR sample was associated with a blood sample. Since the amplification efficiency was not known, the assumption of identical amplification efficiency 100% was made, allowing a more simple quantification model [15].

The Ct values of each gene were translated to normalized expression quantities using two reference genes in a form of normalization index. The normalization index was calculated as an arithmetic mean of the Ct values of the two reference genes:

$$\text{Reference index} = \text{mean}(Ct_{UBC}, Ct_{GAPDH}) \qquad (1)$$

Then, an expression of every target gene was calculated relatively to the expression of the housekeeping gene as

$$\text{Normalized expression} = \frac{2^{\text{reference index}}}{2^{\text{Ct target gene}}} \qquad (2)$$

where the 2 represents the 100% amplification efficiency. The normalized expressions of the time points 2, 16 and 90 days were then

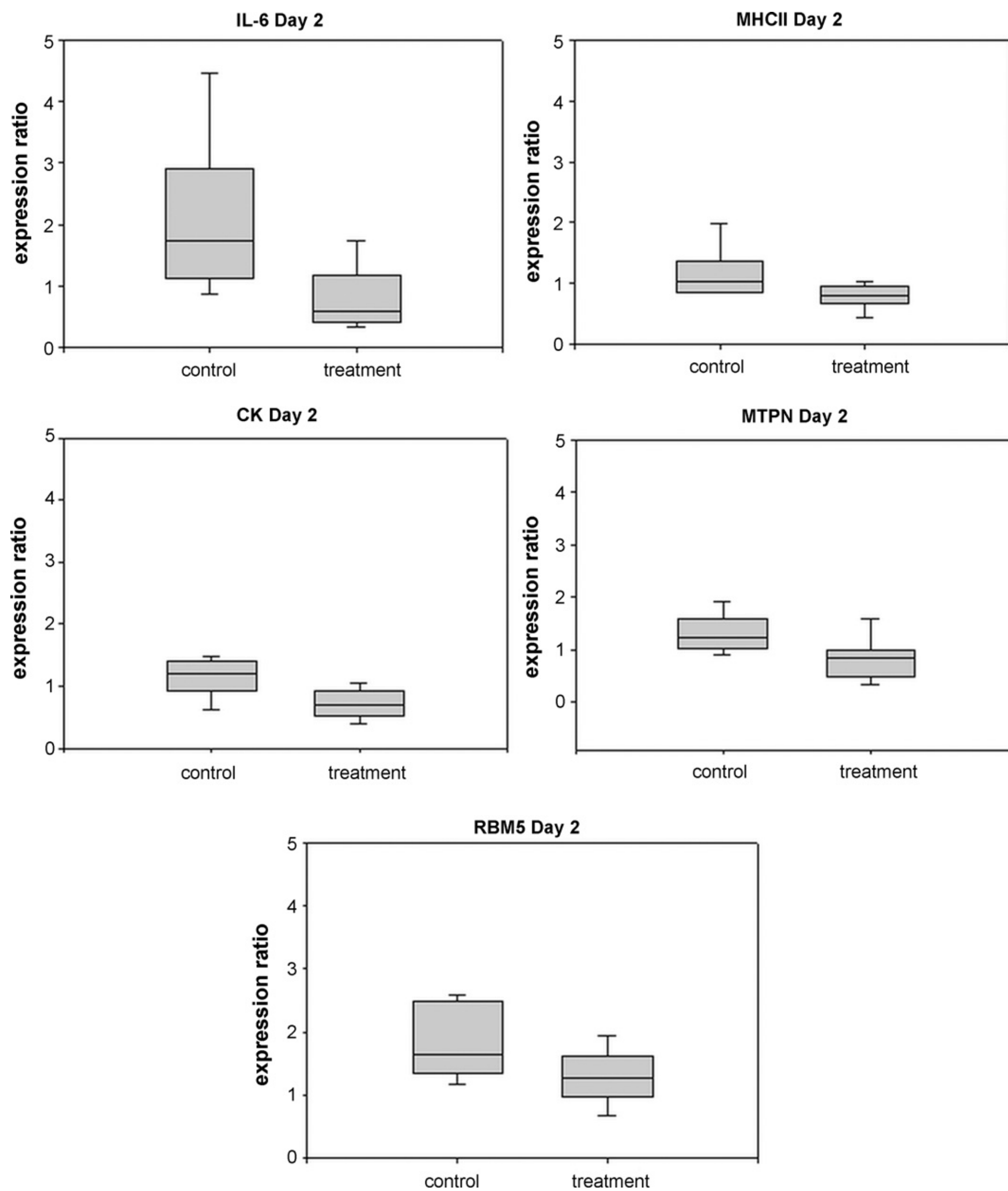

Fig. 1. Significant regulation for IL-6 (A), MHCII (B), CK (C), MTPN (D), and RBM5 (E) between control and treated samples after 2 days of treatment. Box plots show the group median and standard deviations.

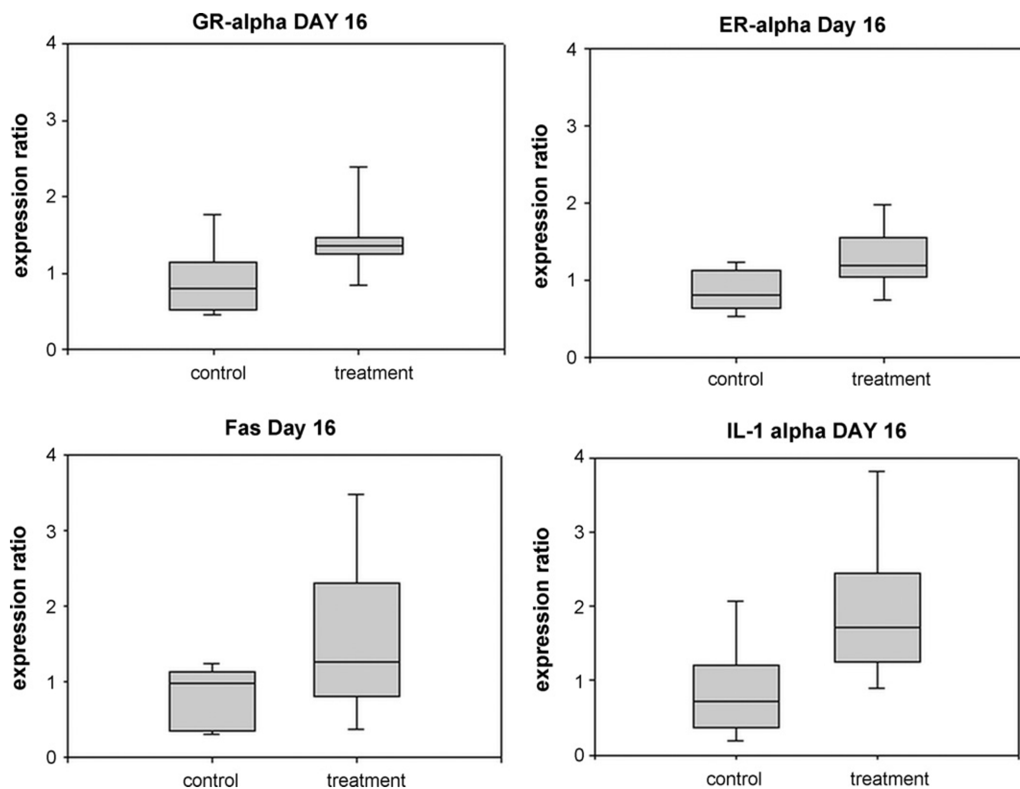

Fig. 2. Significant regulation for GR-α (A), ER-α (B), Fas (C) and IL-1α (D) between control and treated samples after 16 days of treatment. Box plots show the group median and standard deviations.

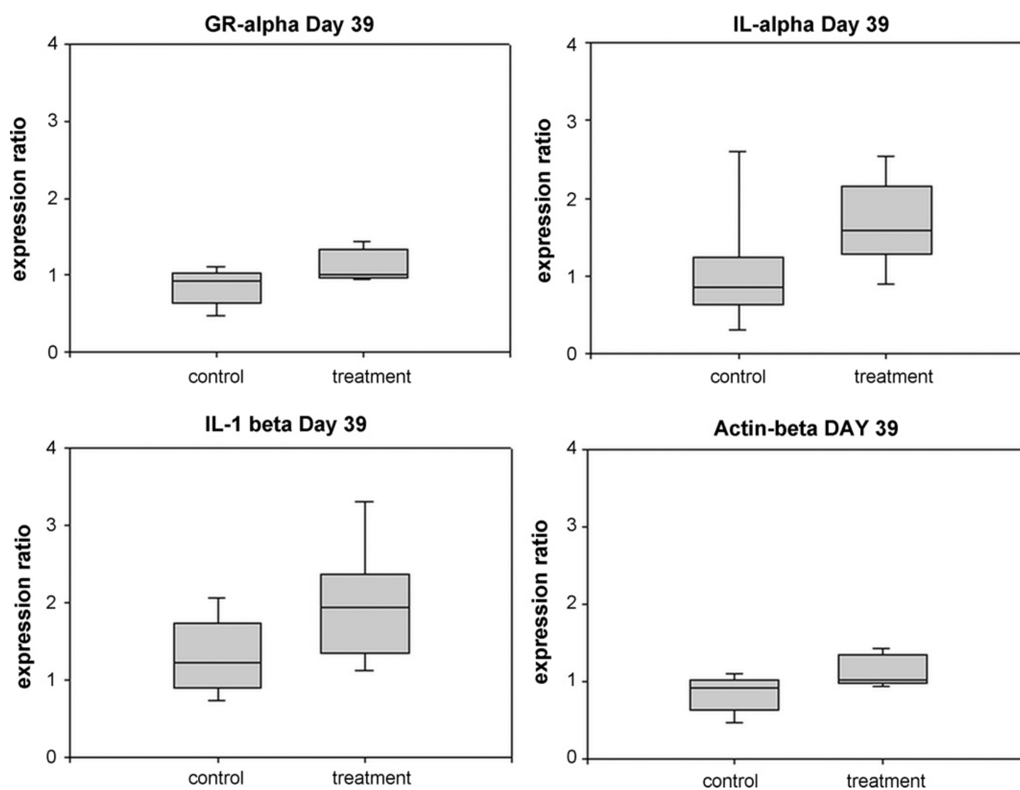

Fig. 3. Significant regulation for GR-α (A), IL-1α (B), IL-1β (C) and Actin-β (D) between control and treated samples after 39 days of treatment. Box plots show the group median and standard deviations.

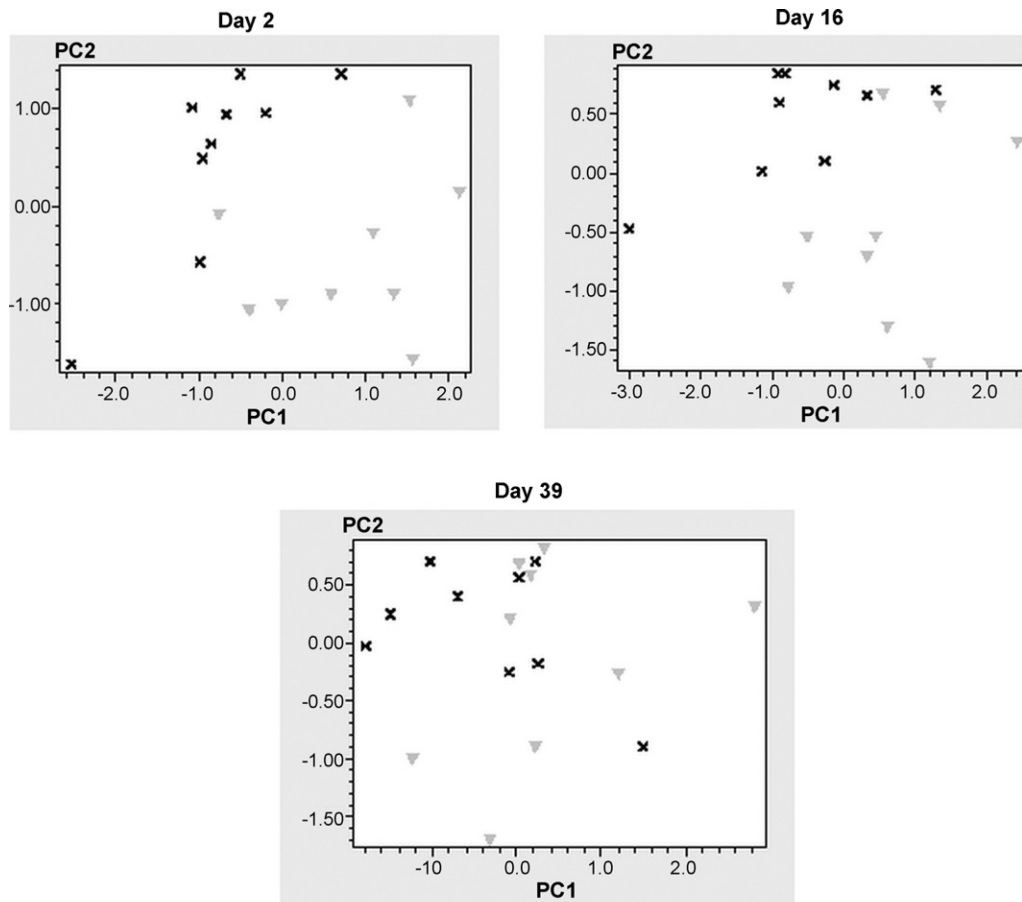

Fig. 4. Principal components analysis (PCA) for the eleven regulated genes GR-α, ER-α, Fas, IL-1α, IL-1β, IL-6, MHCII, CK, MTPN, RBM5 and Actin-β at the three different treatment time points. Animals of the control groups are represented by grey dots and animals of the treatment group are represented by black dots.

divided with the normalized expressions of the baseline (predose), generating the expression ratio R as

$$R_{\text{timepoint/baseline}} = \frac{\text{Normalized expression}_{\text{timepoint}}}{\text{Normalized expression}_{\text{baseline}}} \qquad (3)$$

The expression ratio R was then analysed statistically using the t-test. The Box-whisker plot was constructed to facilitate visual screening of regulated genes (Figs. 1–3).

The objective of the statistical analysis was to disclose genes with significant regulation between control group and treatment group. Hence, this study is to be considered as purely explorative whereas significant findings here indicate candidate biomarkers.

To disclose multivariate response to the treatment, the method of principal component analysis (PCA) was employed using GenEx v. 4.3.6 (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. Normalized expression values of all responding genes were taken as the initial variables and reduced to two principal components only, facilitating thus resolution of treatment clusters in the scatter plot (Fig. 4) [12].

3. Results and discussion

3.1. RNA integrity

Good RNA quality is important for the overall success of RNA based analysis methods like real time qRT-PCR [7,8,21,25]. The RNA degradation level was determined using the lab-on-a-chip technology of the Agilent Bioanalyzer 2100 (Agilent Technologies). The mean (±std. dev.) RIN value of the blood samples was 8.3 ± 0.3 indicating fully integer total RNA.

3.2. Primer testing and gel electrophoresis

Primer pairs of 38 genes were successfully used in quantitative RT-PCR analysis to get single peaks and uniform melting curves.

Table 2
List of p values for the regulation of the amount of the different blood cells.

Time point	White blood cell count	Lymphocytes	Monocytes	Eosinophils	Basophils
Predose	0.5347	0.9263	0.1273	0.1914	0.1691
Day 2	0.2827	0.8051	0.8979	0.3663	–
Day 16	0.9310	0.7601	0.0848	0.3551	0.3927
Day 39	0.3758	0.5106	0.4026	0.0690	0.8353

Table 3
Significant mRNA expression changes. *p* values and *x*-fold regulation between steroid treatment and control group.

Gene group	Gene	Time point	*p* value	Fold regulation
Steroid receptors	GR-a	Day 16	0.0159	1.597
		Day 39	0.0273	1.345
	ER-a	Day 16	0.0106	1.509
Apoptosis regulators	Fas	Day 16	0.0463	1.978
Interleukins	IL-1a	Day 16	0.0108	2.268
		Day 39	0.0364	1.650
	IL-1b	Day 39	0.0412	1.475
	IL-6	Day 2	0.0125	0.434
Others	MHCII	Day 2	0.0219	0.682
	CK	Day 2	0.0046	0.637
	MTPN	Day 2	0.0129	0.621
	RBM5	Day 2	0.0353	0.637
	Actin-b	Day 39	0.0095	1.345

3.3. Haemogram

The haemograms indicate that the animals were healthy. The white blood cell count and the amount of lymphocytes, monocytes, eosinophil, and basophil granolucytes ranged in physiological levels with no significant changes between both treatment groups (*p* values are listed in Table 2). Therefore significant changes in mRNA expression can be interpreted as real changes in gene expression and are not due to changes in the blood cell, especially the mRNA expressing white blood cells.

3.4. qRT-PCR results and data analysis

Significant regulation of gene expression of the treatment group compared to the control group could be identified for IL-6, MHC II, CK, MTPN and RBM5 after 2 days (Fig. 1), for GR-α, ER-α, Fas and IL-1α after 16 days (Fig. 2) and for Actin-β, GR-α, IL-1α and IL-1β after 39 days of treatment (Fig. 3). The resulting *p* values and the regulation ratio between control and treatment are listed in Table 3.

In the box-whisker plots it can be observed that there are also differences of gene expression in the control group compared to baseline. This reflects the natural variability of the non-induced expression in each studied subject.

The number of quantified genes was yet too less to draw conclusions on the different pathways but anyhow first physiological declarations can be made and genes that could act as potential biomarkers could be identified.

The steroid receptors GR-α and ER-α show an up-regulation in the treatment group compared to the control. GR-α is up-regulated at day 16 and day 39 whereas ER-α is only up-regulated at day 16. Trenbolone acetate has an antiglucocorticoid effect via binding to the glucocorticoid receptor [2,17,22]. It is already shown that anabolic steroids influence the mRNA expression of GR-α and ER-α in muscle tissue [24]. The applied hormone combination acts via both regulated steroid receptors. The up-regulation of both receptors indicate that in white blood cells the expression of these receptors is stimulated by its ligands.

The interleukins IL-1α and IL-1β are up-regulated. IL-1α is up-regulated at day 16 and day 39 whereas IL-1β is only regulated after 39 days of treatment. IL-1α and IL-1β are produced by macrophages, monocytes and dendritic cells. During infection they induce the release of other cytokines. The expression of IL-1β can be induced by IL-1α. This could be an explanation why IL-1α is up-regulated after 16 days of treatment whereas IL-1β is only up-regulated after 39 days of treatment [5,6].

Principal components analysis (PCA) is a technique used to reduce multidimensional data sets to lower dimensions for analysis. This statistical method was used to determine whether there is a clustering between control and treatment group. Fig. 4 was obtained by plotting all samples of the two groups in the different time points by their two principal components obtained from the 11 regulated genes. Each group was marked by a color. Black crosses represent samples of the control group and grey triangles show the samples of the treatment group. At days 2 and 16 of treatment it can be observed that both group arrange together and that a difference between control and treatment group can be monitored.

This observation is a first hint that it is possible to get a gene expression pattern opening the possibility to develop a screening method to control the misuse of anabolic hormones in cattle via blood cells. It will be a question of further in vivo trials to determine, if the suggested parameters are independent of breed, nutrition, age, gender and immune status of the animals, and whether they are sensitive enough to uncover low dosages.

4. Conclusions

This pilot study demonstrates that gene expression analysis could be a promising complement to hormone residue analysis for surveillance of hormone misuse in animal production. It could be shown that the combination of trenbolone acetate plus estradiol influences gene expression of 11 genes out of 38 tested candidate genes. Using principle component analysis such regulated genes could act as first biomarkers to discover the illegal use of anabolic hormones in cattle.

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Appendix V

The potential of bovine vaginal smear for biomarker development to trace the misuse of anabolic agents

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29 **Abstract**
30
31 In the European Union the use of anabolic hormones in meat production is forbidden since
32 1988 and this ban of anabolic agents in animal production is strictly controlled. New
33 hormone cocktails passing the detection systems are attractive for the practice and so new
34 approaches to discover their illegal use have to be developed steadily. Verifying
35 physiological effects caused by anabolic steroids will be a new way to develop potential
36 monitoring systems. One promising tissue in female animals will be vaginal smear
37 containing vaginal epithelial cells, because the vaginal epithelium is a primary steroid
38 hormone responsive organ.
39 In this study, we quantified the gene expression in bovine vaginal smear in order to observe
40 physiological effects. Further we aimed to establish a new screening method using
41 physiological regulations of mRNA expression of selected genes caused by anabolic steroid
42 hormones.
43 In an animal trial 9 Nguni heifers were treated with the anabolic combination trenbolone
44 acetate plus estradiol. Vaginal smear samples were taken before treatment (predose), at
45 day 2, at day 16 and at day 39 of treatment. Gene expression of 27 candidate genes,
46 selected by screening the actual literature for steroidal effects on vaginal epithelial cells,
47 were estimated using quantitative real-time RT-PCR.
48 It could be shown that the anabolic combination trenbolone acetate plus estradiol
49 significantly influenced the expression of the steroid receptor ERα, the keratinization factor
50 CK8, the proinflammatory interleukins IL-1α and IL-1β, the growth factors FGF7, EGF,
51 EGFR, IGF-1R, TGFα and LTF, the oncogen c-jun and other factors like actinβ and ubiquitin
52 3.
53 Using biostatistical tools like principal components analysis or hierarchical cluster analysis,
54 the potential to develop a gene expression pattern for targeting the illegal use of growth
55 promoters could be demonstrated.
56
57 **Keywords:** anabolic agents, trenbolone acetate, estradiol, gene expression biomarker, real-
58 time qRT-PCR
59

Introduction

The use of growth promoters like anabolic steroids and β-agonists in meat production is approved e.g. in the USA, Canada or South Africa. These agents are efficient to improve weight gain and feed efficiency in meat producing animals in order to increase the productivity and to reduce costs [1,2]. In the European Union the use of growth promoters in animal husbandry is forbidden because of potential adverse effects of hormone residues to the consumer [3,4]. To prevent the misuse of anabolic agents permanent control is essential [1,3,5-7]. In practice immuno assays or chromatographical methods in combination with mass spectrometry are used to detect hormone residues [8-11]. To mask a proper detection by conventional screening methods, hormone cocktails are applied with doses of each single hormone being below the detection limit [12,13]. Therefore it is necessary to develop new monitoring systems to detect a whole class of anabolic drugs at low concentrations.

Verifying physiological effects caused by anabolic steroids will be a new way to develop potential monitoring systems. One promising tissue in female animals will be vaginal smear containing vaginal epithelial cells, because the vaginal epithelium is a primary hormone responsive organ [14] and vaginal smear can be easily taken in a non-invasive form from the living animal. A potential way to verify physiological effects of steroids will be monitoring of changes in mRNA gene expression [15,16]. A specific and sensitive method to quantify changes in mRNA expression is quantitative real-time RT-PCR [17].

In this pilot study heifers were treated with an anabolic combination used in practice in countries where the application of growth promoters in animal husbandry is permitted. This known preparation was used, because it should be ensured that an anabolic effect occurs. The aim of this study was to test if gene expression changes caused by anabolic agents can be monitored in vaginal smear via RT-qPCR. A second point was to test if these changes could act as potential biomarkers for the effective control of the misuse of anabolic agents.

86 **Experimental**

87

88 ***Animal experiment***

89 18 healthy, non pregnant, two year old Nguni heifers were equally separated to two groups of
90 nine animals each. One group was treated with Revalor H® (140mg trenbolone acetate plus
91 14mg estradiol; Intervet, Isando, RSA) by implantation into the middle third of the pinna of
92 the ear and one group remained untreated serving as control.
93 Vaginal smear samples were taken at four time points: predose samples five days before
94 treatment, and at day 2, day 16 and day 39 of treatment. Vaginal smear, which contains
95 epithelial cells, was taken using a sterilized spoon. The smear was then directly transferred
96 into TriFast (Peqlab Biotechnologies, Erlangen, Germany) and stored at -80°C.
97 To display the cyclic level of the animals at the sampling time points, plasma progesterone
98 was determined 5 days before treatment start, and at day 2, day 16 and day 39 of treatment.
99 The animal attendance, blood sampling and determination of plasma progesterone were
100 done by the Onderstepoort Veterinary Institute (Onderstepoort, Pretoria, South Africa). The
101 animals were housed and fed according to common practice and the South African law.

102

103 ***Total RNA extraction and quality analysis***

104 RNA from vaginal epithelial cells was extracted using peqGold TriFast (PeqLab
105 Biotechologies) according to the manufacturer´s instructions. To quantify the amount of total
106 RNA extracted, optical density (OD) was measured with the NanoDrop 1000 (Peqlab
107 Biotechnologies) for each sample. RNA purity was calculated using the $OD_{260/280}$ratio.

108 RNA integrity and quality control was performed via capillary electrophoresis in the
109 Bioanalyzer 2100 (Agilent Technology, Palo Alto, USA). Eukaryotic total RNA Nano Assay
110 was taken for sample analysis and the RNA Integrity Number (RIN) served as RNA quality
111 parameter. Agilent 2100 Bioanalyzer calculated the RIN value based on a numbering system
112 from 1 to 10 (1 being the most degraded profile, 10 being the most intact) [18].

113

114 ***RNA reverse transcription***

115 Constant amounts of 500ng total RNA were reverse transcribed to cDNA using the following
116 master mix: 12µL 5×Buffer (Promega, Mannheim, Germany), 3µL Random Hexamer Primers
117 (50 mM; Invitrogen, Carlsbad, USA), 3µL dNTP Mix (10 mM; Fermentas, St Leon-Rot,
118 Germany) and 200 U of MMLV Reverse Transcriptase (Promega) according to the
119 manufacturer's instructions.

120

121

122 ***Specific primer design***

123 All primers were designed using published bovine nucleic acid sequences in GenBank
124 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Primer design and optimization was done
125 with primer design program of MWG Biotech (MWG, Ebersberg, Germany) and primer3
126 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with regard to primer dimer and
127 self-priming formation. Newly designed primers were ordered and synthesized at MWG
128 Biotech. Primer testing was performed with three optional samples and a no template control
129 (NTC contains only RNAse free water). To determine the optimal annealing temperature for
130 each primer set a temperature gradient PCR was done. All used primers are listed in table 1.
131

132 ***Quantitative real-time PCR analysis***

133 To analyze gene expression of candidate genes, qPCR analysis was done using the IQ5
134 (Bio-Rad, Munich, Germany). Quantitative real-time PCR was performed using MESA
135 GREEN qPCR MasterMix Plus for SYBR® Assay w/ fluorescein Kit (Eurogentec, Cologne,
136 Germany) by a standard protocol, recommended by the manufacturer.
137 With the kit the master mix was prepared as follows: For one sample it is 7.5µL MESA
138 GREEN 2x PCR Master Mix, 1.5µL forward primer (10 pmol/µL), 1.5 µL reverse primer (10
139 pmol/µL) and 3µL RNase free water. For qPCR analysis 1.5µL cDNA was added to 13.5µL
140 Master Mix. qPCR was performed in 96 Well Plates (Eppendorf, Hamburg, Germany) and
141 pipetting was done by the epMotion 5075 (Eppendorf).
142 The following general real-time PCR protocol was employed for all investigated factors:
143 denaturation for 5 min at 95°C, 40 cycles of a two segmented amplification and quantification
144 program (denaturation for 3 s at 95°C, annealing for 10 s at primer specific annealing
145 temperature), a melting step by slow heating from 60 to 95°C with a dwell time of 10 s and
146 continuous fluorescence measurement. Threshold cycle (Ct) and melting curves were
147 acquired by using the iQ5 Optical System software 2.0 (Bio-Rad). Only genes with clear
148 melting curves were taken for further data analysis. Samples that showed irregular melting
149 peaks were excluded from the quantification procedure.
150

151 ***Selection of target genes***

152 Candidate genes that might be biomarkers in vaginal epithelial cells were chosen by
153 screening the respective literature for steroidal effects on vaginal epithelial cells. It is known
154 that the expression and mRNA stabilization of the different steroid receptors is influenced by
155 their ligands [19-23]. Therefore the steroid receptors androgen receptor (AR), estrogen receptor
156 alpha (ERα) and beta (ERβ) and the progesterone receptor (PR) were chosen as candidate
157 genes. Estrogens regulate the proliferation and keratinization of vaginal epithelial cells.
158 Therefore following candidate genes were chosen for quantification: the keratinization factors

159 cytokeratinase 8 (CK8) and cytokeratinase 18 (CK18) [22,24], the growth factors fibroblast
160 growth factor 7 (FGF7), fibroblast growth factor binding protein (FGFBP) epidermal growth
161 factor (EGF) its receptor EGFR, tumor growth factor α (TGFα), insulin like growth factor 1
162 (IGF-1), its receptor IGF-1R, the insulin like growth factor binding protein 3 (IGF-BP3) and
163 lactoferrin (LTF) [22,24-26], and in addition the apoptosis regulators TNF receptor superfamily
164 member 6 (Fas), its ligand FasL, tumor necrosis factor receptor 1 (TNFR1), its ligand tumor
165 necrosis factor α (TNFα), Caspase 3 (Casp 3) and Caspase 8 (Casp 8) [22] and the
166 proinflammatory interleukins (IL) IL-1α and IL-1β [24]. Further genes that were quantified were
167 the oncogenes c-jun and c-fos [22] and the lingual antimicrobial peptide (LAP). As reference
168 gene candidates Ubiquitin 3 (UB3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH),
169 actinβ (ACTB), histone H3 and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase
170 activation protein, zeta polypeptide (YWHAZ) were measured, whereas histone H3 and
171 YWHAZ were chosen as best reference genes by using GenEx Ver 4.3.6 Software (MultiD
172 Analyses AB, Gothenburg, Sweden).
173
174
175 ***Data Analysis and Statistics***
176 Statistical description of the expression data as well as statistical tests were produced with
177 Sigma Stat 3.0 (Systat Software GmbH, Erkrath, Germany). The raw data were the Ct values
178 obtained from each qPCR sample. Each qPCR sample was associated with a vaginal smear
179 sample. Since the amplification efficiency was not known, the assumption of identical
180 amplification efficiency 100% was made, allowing more simple quantification model [27].
181 The Ct values of each gene were translated to normalized expression quantities using two
182 reference genes in a form of normalization index. The normalization index was calculated as
183 an arithmetic mean of the Ct values of the two reference genes histone and YWHAZ
184 (equation 1):
185
186 reference gene index = mean ($Ct_{histone}$, Ct_{YWHAZ}) [1]
187
188 Then, an expression of every target gene was calculated relatively to the expression of the
189 housekeeping gene as (equation 2):
190
191 normalized expression (N) $= 2^{\text{reference gene index}} / 2^{\text{Ct target gene}}$, [2]
192
193 where the 2 represents the 100% amplification efficiency in each performed PCR cycle.
194

195 The normalized expression was then analyzed statistically using the t-test (Sigma Stat 3.0,
196 Systat Software GmbH). The objective of the statistical analysis was to disclose genes with
197 significant regulation between control group and treatment group. Hence, this study is to be
198 considered as purely explorative whereas significant findings here indicate candidate
199 biomarkers.
200 To disclose multivariate response to the treatment, the method of principal components
201 analysis (PCA) was employed using GenEx version 4.3.6 (MultiD Analyses AB). PCA
202 involves a mathematical procedure that transforms a number of variables (here normalized
203 expression values) into a smaller number of uncorrelated variables called principal
204 components. By this the dimensionality of the data is reduced to a number of dimensions that
205 can be plotted in a scatter plot, here two dimensions. The first principal component accounts
206 for as much of the variability in the expression data as possible, and each succeeding
207 component accounts for as much of the remaining variability as possible. Normalized
208 expression values of all responding genes were taken as the initial variables and reduced to
209 two principal components only, facilitating thus resolution of treatment clusters in the scatter
210 plot [28].
211 Another method for visualizing treatment patterns based on multivariate data is hierarchical
212 cluster analysis. The hierarchical order is represented by a tree dendogram, in which related
213 samples are more closely together than samples that are more different. Hierarchical
214 clustering was employed using GenEx v. 4.3.6 (MultiD Analyses AB).

215 **Results and Discussion**

216

217 ***Plasma progesterone levels***

218 To determine the estrous state of the animals, plasma progesterone levels were determined
219 at all four sampling time points. The average progesterone levels of the two groups at the
220 different time points and the standard deviations are shown in table 2.
221 Before treatment start mean plasma progesterone and the standard deviation of both groups
222 were relatively high. In the control group, this effect persisted during the whole trial. At days
223 2, 16 and 39 of treatment, mean plasma progesterone levels and the standard deviation of
224 the treatment group were much lower than in the control group. This indicates that the
225 treatment down-regulates the estrous cycle of the animals. The high standard deviations in
226 the control group denotes, that the control animals were at different stages of the estrous
227 cycle, when vaginal smear samples were taken. This is of advantage, because by this means
228 there is high variability in natural estrogen levels in the control group and changes in gene
229 expression quantified between the control group and the treatment group are real effects of
230 the treatment and do not occur due to low estrogen levels in all animals of the control group.
231 Used in practice as a screening method, tested animals will also be at different stages of the
232 estrous cycle. Though untreated control samples used in treatment screening have to be
233 obtained from animals at different stages of the estrous cycle to exclude the influence of
234 natural variability of estrogen level during the estrous cycle on the test results.
235 The observation that progesterone levels of treated animals is lower than that of the
236 untreated animals could act as an additional indicator for anabolic treatment in heifers. Using
237 progesterone levels as single indicator, progesterone levels would have to be determined
238 several times over a long period for each animal. Furthermore there are also other factors
239 that could result in a low progesterone level. Twin born female calves for example could have
240 lost ovaries and so no progesterone is released. But in combination with other parameters,
241 like gene expression analysis, the determination of blood progesterone could act as potential
242 indicator for the use of anabolic steroids.

243

244 ***RNA Integrity, primer testing and gel electrophoresis***

245 The RNA degradation level was determined using the lab-on-a-chip technology of the Agilent
246 2100 Bioanalyzer. The RIN value of the vaginal smear samples was 4.5 ± 2.02 (mean ± SD).
247 The relatively low RNA quality could be due to the fact that cells found in the vaginal smear
248 are detached, keratinized and partly degraded. Another reason for the low RNA quality
249 results can be RNases present in the vaginal flora. But quantitative analysis via real-time RT-
250 PCR measurements worked very well and primer pairs of 29 genes (27 candidate genes and
251 2 reference genes) were successfully used in qRT-PCR analysis to get single peaks and

252 uniform melting curves, as well as a specific single band in high resolution agarose gel
253 electrophoresis.
254

255 ***RT-qPCR results and data analysis***

256 The steroid receptor ERα showed a significant down-regulation after two days of treatment
257 (p=0.046). Hormones regulate the concentrations of their receptor proteins. Either they do it
258 by regulating the transcription of the receptor gene or by regulating the stability of the
259 receptor mRNA [20]. The observed down-regulating effect of estrogens on the estrogen
260 receptor was already reported for vaginal cells of mice and rats [19,22].
261 The keratinization factor CK8 (p=0.003) was significantly down-regulated after two days of
262 treatment. CK8 is preferentially expressed in epithelial cells, e.g. in vaginal epithelium. In
263 mice it was already shown that estrogens down regulate the mRNA expression of CK8 [24].
264 The growth factors FGF7 (p=0.009), EGF (p=0.005), EGFR ($p=0.5\times10^{-4}$), TGFα ($p=0.5\times10^{-3}$
265), IGF-1R (p=0.007) and LTF (p=0.031) were significantly regulated, whereas EGF, TGFα,
266 IGF-1R were down-regulated at day 2, FGF7 and EGFR were up-regulated after 16 days and
267 LTF was up-regulated after 39 days of treatment. Factors that are involved in the stimulation
268 of the proliferation of epithelial cells are the growth factors FGF7, EGF and EGFR [22,29,30].
269 FGF7 and EGF stimulate epithelial growth in vaginal epithelium in mice [24,31,32]. The effect of
270 estrogens on mRNA expression of these three factors was already shown in mice vaginal
271 epithelial cells [22,31-33]. The regulation of the growth factors IGF-1R and LTF also goes in line
272 with effects of estrogens that could already be shown in mice. Miyagawa et al (2004)
273 reported, that the mRNA expression of members of the IGF Family is regulated by
274 diethylstilbestrol, a synthetic nonsteroidal estrogen [24]. In this study the down-regulating effect
275 of estrogens on IGF-1R could also be observed. Sato et al (2004) demonstrated that
276 neonatal exposure of mice with diethylstilbestrol results in an up-regulation of EGF and LTF
277 [22,33]. It is already known that estrogen stimulates LTF mRNA expression in uterine tissue [25,34]
278 and that LTF is present at various stages of the estrus cycle in human uterus and vaginal
279 epithelium [25,35,36]. This study shows that LTF mRNA expression is increased by estrogen
280 treatment in the bovine vaginal epithelium.
281 Other factors of which the mRNA expression level was changed are the pro-inflammatory
282 interleukins IL-1α (p=0.016) and IL-1β (p=0.005) (both down-regulated after 39 days of
283 treatment) and the oncogen c-jun (p=0.005) (down-regulation at day 2). Furthermore ACTB
284 (down-regulation at day 2, p=0,007) and UB3 (down-regulation at day 2, p=0.018, and day
285 16, p=0.001) were significantly regulated. The expression ratios are listed in table 3.
286 The second aim of this study was to investigate whether the observed changes of mRNA
287 expression can act as biomarkers to develop a screening method for the combination of
288 trenbolone acetate plus estradiol.

289 Principal components analysis (PCA) was used to determine whether there is a clustering
290 between control and treatment group. Figure 1 was obtained by plotting all samples of the
291 two groups in the different time points by their two principal components obtained from the 13
292 significantly regulated genes. Some genes showed no significant regulation but showed a
293 trend to be regulated ($p<0.1$) Therefore PCA was also done by plotting all samples of the two
294 groups in the different time points by their two principal components obtained from all 27
295 measured candidate genes. Each group was marked by color. Black crosses represent
296 samples of the control group and grey triangles show the samples of the treatment group. At
297 all three treatment time points it can be observed that both groups arrange together and that
298 a difference between control and treatment group can be monitored. Before treatment the
299 groups show no difference in gene expression of analyzed target genes. This effect is better
300 visible using all 27 quantified genes.
301 Another biostatistical method to visualize whether the groups arrange together is Hierarchical
302 Cluster Analysis. To verify if the effect observed by PCA is also visibly by using this method,
303 hierarchical clustering was done with the data of the day 16 samples obtained from all
304 measured genes (Figure 3). The dendogram shows a clear separation between the two
305 groups by showing two main branches. The one above only represents untreated samples.
306 The other one represents treatment samples exept of sample Revalor H 6. Performed as
307 treatment screening this sample would be a false positive one.
308
309
310
311

312 **Conclusions**

313 In this study the potential of gene expression analysis in vaginal smear for developing a new
314 screening method to trace the abuse of anabolic steroid hormones is examined.
315 Although the quality of RNA obtained from bovine vaginal smear is poor, gene expression
316 data in combination with PCA or hierarchical cluster analysis show promising results for the
317 development of potential gene expression biomarkers. Both biostatistical methods show a
318 clear clustering of the treatment groups.
319 To confirm these results, new studies using anabolic agents in concentrations under the
320 detection limit of conventional methods will be helpful.
321

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389 Table 1:
390

Gene group	Gene		Sequence	Annealing Temperature	Product length
Steroid Receptors	AR	for	CCT GGT TTT CAA TGA GTA CCG CAT G	60°C	172bp
		rev	TTG ATT TTT CAG CCC ATC CAC TGG A		
	ERα	for	AGG GAA GCT CCT ATT TGC TCC	60°C	233bp
		rev	GGT GGA TGT GGT CCT TCT C		
	PR	for	ACC AGC CCT ATC TCA ACT ACC	60°C	186bp
		rev	TAT GCT GTC CTT CCA TTG CCC		
Keratinization Factors	FGF7	for	GACATGGATCCTGCCAAGTT	60°C	129bp
		rev	GGGCTGGAACAGTTCACATT		
	FGFBP	for	CTT CAC TGG CAA TCC AAC CTC	60°C	116bp
		rev	AGG ACA CTC TTG GCA TCA CC		
	CK8	for	GCT ACA TTA ACA ACC TCC GTC G	60°C	237bp
		rev	TCT CAT CAG TCA GCC CTT CC		
	CK18	for	TCG ATG ACA CCA ATG TCA CCC	60°C	249bp
		rev	ACC AGT ACT TGT CCA GCT CC		
Growth Factors	EGF	for	TGC TGT CTC ACC TTG GGA AG	64°C	136bp
		rev	CAG GAG AAC AGG TTG GCA ATG		
	EGFR	for	TTC ACA CGT ACT GCA CCT CTG	60°C	243bp
		rev	CAC ATC ACC ATC ACT TAT CTC C		
	IGF-1	for	CAT CCT CCT CGC ATC TCT TC	60°C	238bp
		rev	CTC CAG CCT CCT CAG ATC AC		
	IGF-1R	for	TTA AAA TGG CCA GAA CCT GAG	62°C	314bp
		rev	ATT ATA ACC AAG CCT CCC AC		
	IGFBP3	for	ACA GAC ACC CAG AAC TTC TCC T	63°C	202bp
		rev	AGA AAC CCC GCT TCC TGC C		
	TGFα	for	TGA CTG CCC AGA TTC CCA CA	64°C	238bp
		rev	GCA GCA GTG TAT CAG CAC ACA		
	LTF	for	CGA AGT GTG GAT GGC AAG GAA	60°C	222bp
		rev	TTC AAG GTG GTC AAG TAG CGG		
Apoptosis	Fas	for	TGT TGT CAG CCT TGT CCT CC	60°C	174bp
		rev	GTT CCA CTT CTA GCC CAT GTT C		
	FasL	for	CAT CTT TGG AGA AGC AAA TAG	60°C	205bp
		rev	GGA ATA CAC AAA ATA CAG CCC		
	Casp 3	for	TGC AGA AGT CTG ACT GGA AAA CCC AAA C	60°C	101bp
		rev	TCA TCC TCA GCA CCA CTG TCT GTC TC		
	Casp 8	for	TAG CAT AGC ACG GAA GCA GG	60°C	294bp
		rev	GCC AGT GAA GTA AGA GGT CAG		
	TNFα	for	CCA CGT TGT AGC CGA CAT C	60°C	155bp
		rev	CCC TGA AGA GGA CCT GTG AG		
	TNFR1	for	TCC AGT CCT GTC TCC ATT CC	63°C	236bp
		rev	CTG GCT TCC CAC TTC TGA AC		
Interleukins	IL-1α	for	CCT CTC TCT CAA TCA GAA GTC C	62°C	142bp
		rev	CCA CCA TCA CCA CAT TCT CC		
	IL-1β	for	TTC TCT CCA GCC AAC CTT CAT T	60°C	198bp
		rev	ATC TGC AGC TGG ATG TTT CCA T		
Oncogens	c jun	for	TCA ACG CCT CGT TCC TCC	63°C	278bp
		rev	CTC ATC TGT CAC GTT CTT GGG GCA		
	c fos	for	GCT CCA GGC GGA GAC AGA	60°C	302bp
		rev	AGG GTG AAG GCC TCC TCA GA		
Others	UB3	for	AGA TCC AGG ATA AGG GAA GGC AT	60°C	198bp
		rev	GCT CCA CCT CCA GGG TGA T		

	ACTB	for	AAC TCC ATC ATG AAG TGT GAC	57°C	202bp
		rev	GAT CCA CAT CTG CTG GAA GG		
	LAP	for	GAA ATT CTC AAA GCT GCC GTA	60°C	114bp
		rev	TCC TCC TGC AGC ATT TTA CTT		
Reference Genes	histone	for	ACT GCT ACA AAA GCC GCT C	60°C	233bp
		rev	ACT TGC CTC CTG CAA AGC AC		
	YWHAZ	for	CAG GCT GAG CGA TAT GAT GAC	60°C	141bp
		rev	GAC CCT CCA AGA TGA CCT AC		

391

392 Table 1: List of 29 primer pairs used for qRT-PCR analysis.

393 Table 2:
394

treatment group	Predose		Day 2		Day 16		Day 39	
	mean	SD	mean	SD	mean	SD	mean	SD
control	10.48	7.87	12.07	7.57	11.18	12.35	11.68	10.54
treatment	6.04	8.37	4.70	4.17	4.47	7.03	2.10	2.88

395
396 Table 2: Plasma progesterone levels (nmol/L) and standard deviations at investigated
397 sampling times

398 Table 3:
399

Gene Group	Gene	Day 2	Day 16	Day 39
Steroid receptors	ERα	**0.59**		
Keratinization factors	CK8	**0.42**		
growth factors	FGF7		**2.60**	
	EGFR	**0.36**		
	EGF		**2.79**	
	IGF-1R	**0.63**		
	TGFα	**0.25**		
	LTF			**4.35**
Interleukins	IL-1α			**0.34**
	IL-1β			**0.20**
oncogenes	c-jun	**0.61**		
others	ACTB	**0.46**		
	Ubiquitin	**0.64**	**0.31**	

400
401 Table 3: Expression changes. X-fold regulations between treatment and control group of the
402 significant regulated genes at the three treatment time points. X-fold values = 1 means no
403 regulation, values < 1 means down-regulation of that gene and values > 1 means up-
404 regulation of that specific gene.
405
406

407 Figure Legends:

408

409 Figure 1:

410 Principal components analysis (PCA) for the thirteen significantly regulated genes at the four
411 different sampling time points. Animals of the control groups are represented by black
412 crosses and animals of the treatment group are represented by grey triangles.

413

414 Figure 2:

415 Principal components analysis (PCA) for all 27 measured candidate genes at the four
416 different sampling time points. Animals of the control groups are represented by black
417 crosses and animals of the treatment group are represented by grey triangles.

418

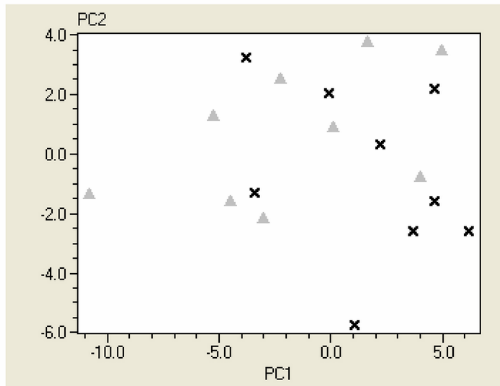
419 Figure 3:

420 Dendogram for all 27 measured candidate genes at sampling time point day 16. Control
421 samples (untreated 1-9) and treated samples (Revalor H) were clustered.

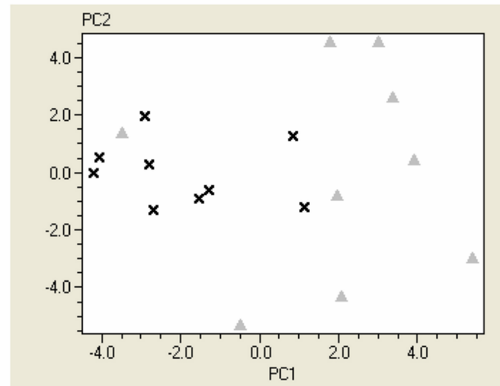
422 Figure 1:

423

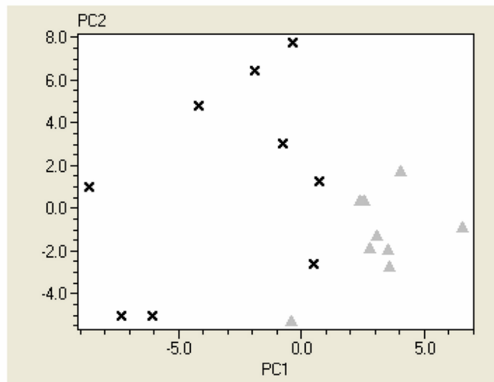
Predose



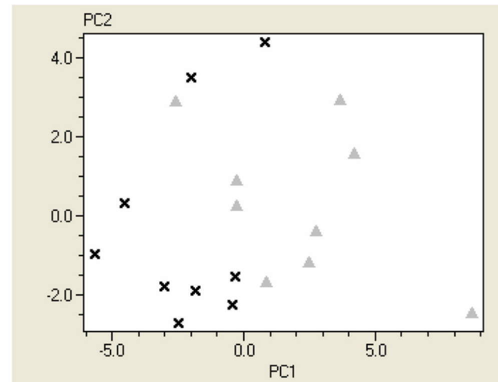
Day 2



Day 16



Day 39



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425 Figure 2:

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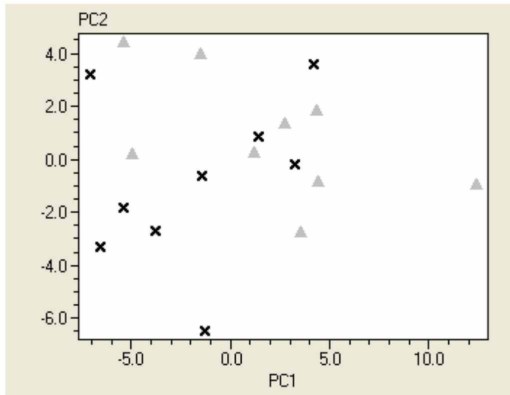
Predose



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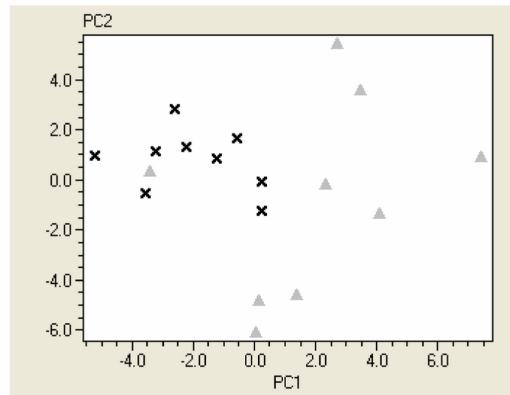

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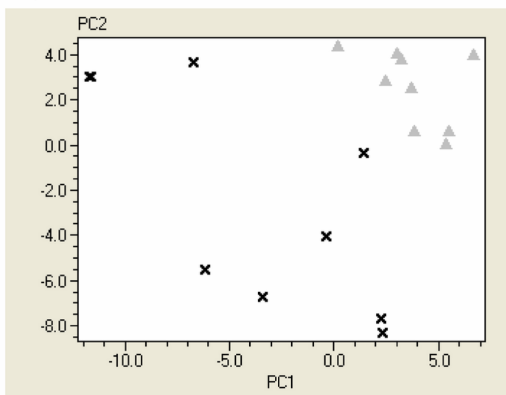
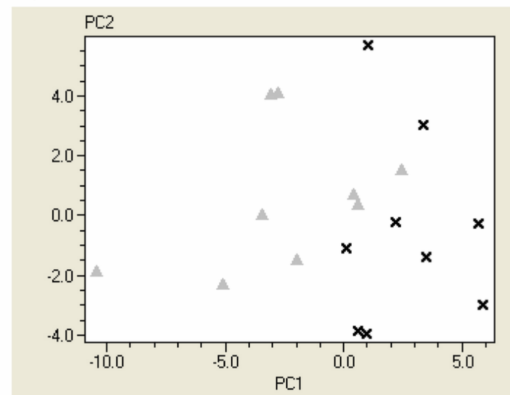


Day 39



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428 Figure 3:
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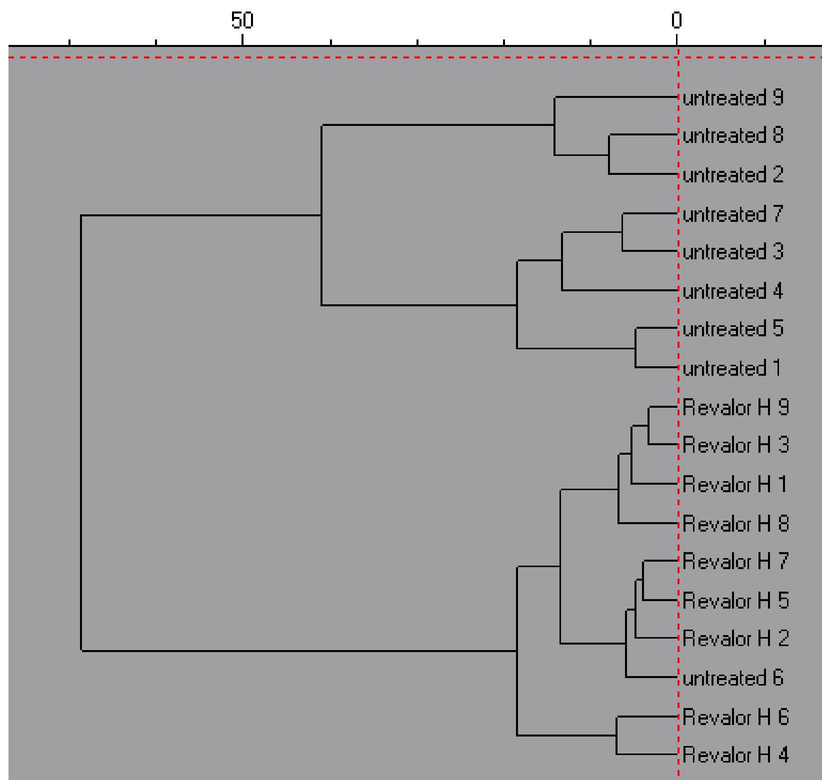

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