Technische Universität München
Lehrstuhl für Physiologie

Modification of metabolic pathways by anabolic agents and identification of gene expression biomarkers

Martina Reiter

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr.rer.nat)

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. Th. Knoke

Prüfer der Dissertation:
1. Univ.-Prof. Dr. H.H.D. Meyer
2. Univ.-Prof. Dr. J. Polster
3. Priv.-Doz. Dr. M. W. Pfaffl

Die Dissertation wurde am 16.04.2008 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 03.07.2008 angenommen.

Wissenschaft –

Einem ist sie die hohe, die himmlische Göttin,
dem Andern eine tüchtige Kuh, die ihn mit Butter versorgt.

(Friedrich Schiller)

TABLE OF CONTENTS

ABBREVIATIONS

AAS	anabolic androgenic substances
ACADvl	acyl-CoA-dehydrogenase
ACC.NR.	accession number
ACTA1	α-actin
ACTB	β-actin
ADI	acceptable daily intake
AM	arithmetic mean
Angpt	angiopoetin
AR	androgen receptor
AT	annealing temperature
bp	base pairs
Bcl-2	b-cell leucemia/lymphoma 2
Bcl-xl	bcl-xl protein
BW	body weight
CALR	calreticulin
CAPN3	calpain 3
CAST	calpastatin
CDH15	m-cadherin
CDK2	cyclin-dependent kinase 2
c-fos	c-fos protein
c-jun	c-jun protein
CK	creatin kinase
Cox	cyclo-oxogenase
CRL	community reference laboratory
Ct	crossing point
CTSB	cathepsin B
CTSL	cathepsin L
C.V.	coefficient of variation
CyP1A1	cytochrome P1
DNA	desoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
e.g.	example given
Enoyl-CoA	enoyl-CoA-hydratase
ER	estrogen receptor
EU	European Union
FAO	food and agriculture organisation
FasL	death receptor ligand
FasR	death receptor
FCS	fetal calf serum
FDA	food and drug administration
FGF	fibroblast growth factor
FGFR	fibrolblast growth factor receptor
FLK-1	vascular endothelial growth factor receptor
for	forward primer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	gas chromatography
GDF8	myostatin
GHR	growth hormone receptor
GLUT	glucose transporter

GR	glucocorticoid receptor
HBSS	hank's buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	hair follicle
HFDPC	hair follicle dermal papilla cells
HGF	hepatocyte growth factor
HK	hexokinase
HRE	hormone responsive element
HSD17b	17β-hydroxy-steroid-dehydrogenase
Hsp	heat shock protein
IGF1	insulin like growth factor 1
IGF1R	insulin like growth factor 1 receptor
IGFBP	insulin like growth factor binding protein
IL	interleucin
IOC	international olympic committee
IR	insulin receptor
JECFA	joint FAO/WHO expert committee on food additives
LDH	lactate-dehydrogenase
MGA	melengestrol acetate
MMP	matrix metalloproteinase
MS	mass spectrometry
Myf	myogenic factor
MYHC2x	myosin heavy chain
MyoD	myogenic differenciation factor
MyoG	myogenin
NCBI	national center for biotechnology information
n.m.	not measured
NRL	national reference laboratory
NT	neurotrophin
OD	optical density
p53	tumor supressor
PCR	polymerase chain reaction
PPIA	cyclophilin A
PRLR	prolactin receptor
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
PYGM	glycogen phosphorylase
rev	reverse primer
RG	reference gene
RGI	RG-Index
RIN	RNA integrity number
RT	reverse transcription
mRNA	messenger ribonucleic acid
SARM	selective androgen receptor modulator
S.D.	standard deviation
s.r.	significantly regulated
SRD5A	steroid-5α-reductase
TAT	tyrosin-amino-transferase
TBA	trenbolone acetate
T/E	testosterone/epitestosterone ratio

TE-buffer	trishydroxymethyl-aminomethane/ ethylene-diaminetetraacetic acid buffer
TIMP	matrix metalloproteinase inhibitor
TG	target gene
T/LH	testosterone/luteinizing hormone ratio
Tm	melting temperature
TM	treatment
TNF	tumor necrosis factor
trt	treatment
TU	technical university
UBC	ubiquitin
USA	United States of America
UV	ultra violet
VEGF	vascular endothelial growth factor
v-myb	myeloblastosis viral oncogene
WADA	world anti doping agency
WHO	world health organisation
YWHAZ	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein
Z	zeranol

ZUSAMMENFASSUNG

Der Begriff anabole Wirkstoffe umfasst alle anabolen androgenen Steroide (AAS) und andere anabole Wirkstoffe. Diese Substanzen werden wegen ihrer anabolen Wirkung eingenommen, welche einen Zuwachs an Muskelmasse, eine Reduzierung der Fettmasse, eine positive Stickstoffbilanz und eine allgemeine Leistungssteigerung bewirken soll.

AAS finden im Leistungssport Anwendung, wobei die Einnahme im Profisport illegal ist und geahndet wird. Bei Lebensmittel liefernden Tieren ist der Einsatz anaboler Substanzen in Ländern wie den USA und Kanada legal, in der EU jedoch verboten. Anabole androgene Substanzen finden auch in der medizinischen Therapie ihren Platz. Hier werden sie überwiegend für die Behandlung von Osteoporose bei Frauen und Hypogonadismus beim Mann eingesetzt. Eine Behandlung mit anabolen Stoffen hat meist auch Nebenwirkungen zur Folge, weshalb neue Substanzen, so genannte SARM (selective androgen receptor modulators) entwickelt wurden. Diese Substanzen sollen dieselbe anabole Wirkung wie das Androgen Testosteron aufweisen, jedoch geringere Nebeneffekte zeigen.

Verschiedenste Methoden um anabole Wirkstoffe nachzuweisen wurden entwickelt, meist basierend auf Massenspektrometrie, welche die direkte Analyse des Wirkstoffes erlaubt. Es ist allerdings auch bekannt, dass anabole Wirkstoffe bereits die mRNA Expression in der Zelle beeinflussen können, weshalb die Wirkung dieser Stoffe mittels mRNA-Expressionsanalysen messbar sein sollte.

Konkrete Zielsetzung dieser Arbeit war es festzustellen, ob AAS mit Hilfe von Gen-Expressionsanalysen in verschiedenen Geweben und Spezies nachzuweisen sind. Durch die Identifizierung von Biomarkern, gemessen mittels qRT-PCR sollte die Entwicklung eines Expressionsmusters, speziell für AAS möglich sein. Ebenso sollten die physiologischen Auswirkungen der Substanzen im Organismus, hervorgerufen durch die anabolen Stoffe aufgezeigt werden. Dafür wurden verschiedene Gewebe, welche alle direkt oder indirekt unter dem Einfluss von anabolen androgenen Stoffen stehen, wie etwa der Uterus, die Leber, Muskeln und Haarfollikel analysiert und nach möglichen Biomarkern und physiologischen Signalwegen geforscht. Die Zielgene wurden in funktionelle Gruppen eingeteilt, um später die Identifizierung metabolischer Veränderung zu erleichtern.

Erste Stoffwechselwege, welche hauptsächlich unter dem Einfluss von anabolen Wirkstoffen zu stehen scheinen, konnten identifiziert werden. Gene des extrinischen Apoptosesignalweges zeigten signifikante Regulationen in allen Geweben und Spezies. Weiter konnte immer auch die Expression der Steroid-Rezeptoren und Enzyme des Energiestoffwechsels unter dem

Einfluss von anabolen Substanzen nachgewiesen werden. Somit war eine Identifizierung erster gewebespezifischer Biomarker möglich, welche wiederum für die Entwicklung eines AAS spezifischen Gen-Expressionsmusters verwendet werden können. Gewebe wie der Uterus, die unter direktem Hormoneinfluss stehen, zeigten sehr hohe Regulationen. Deshalb werden primäre Sexualorgane, wie etwa die Hoden oder der Uterus, aber auch Blut für weitere Versuche empfohlen.

Da es sich bei allen Projekten um Erstversuche handelte, werden weitere Studien nötig sein, um Stoffwechselkaskaden zu identifizieren und weitere Biomarker zu finden, um ein Gen-Expressionsmuster zu vervollständigen. Somit konnten erste Schritte in Richtung einer neuen Nachweismethode für anabole androgene Substanzen gemacht werden, welche zukünftig bei der Aufdeckung von Missbrauchsfällen mit AAS helfen könnte.

ABSTRACT

Anabolic agents are defined as anabolic androgenic substances (AAS) and other anabolic substances. Anabolic agents increase muscle mass, reduce total body fat and induce a positive nitrogen balance, which should result in higher performance.

The use of AAS in competitive sports exists but is illegal. In animal husbandry the application of AAS is legal in the USA and Canada but forbidden in the EU. Also in medical therapies anabolic agents are applied, for example in order to treat osteoporosis in women or hypogonadism in men. Here only the positive effects of the agents are desirable, such as muscle development and an increase in bone density. Unfortunately, the intake of anabolic hormones also includes negative side effects. Therefore special substances called SARM (selective androgen receptor modulators) were developed. SARM should have the ability to mimic the anabolic effects of testosterone with lower negative side effects of the natural hormone.

Various residue analyses for anabolic substances exist and most of them are based on mass spectrometry, which directly analyse the drug. It is also known that AAS can influence mRNA expression. This should make it possible to measure the influence of these substances via gene expression analysis. Taking these substance specific gene expressions the development of a new screening method should be possible.

The aim of this thesis was to proof the possibility to detect AAS treatment via mRNA expression analysis in different tissues and species. With the identification of so-called biomarkers for anabolic agents using qRT-PCR, a gene expression pattern for AAS should be established. The identified gene regulations could also be used to show up metabolic pathways, influenced by the AAS. Therefore various androgen dependent tissues like the uterus, liver, muscle and hair were analysed, to find such biomarkers as well as signalling pathways of the steroids. All target genes were separated into functional groups to facilitate the identification of metabolic influences.

First effects of AAS treatment on metabolic pathways could be analysed. Target genes of the extrinsic apoptosis pathway were significantly regulated in all tissues and species. Additionally the expression of the steroid receptors and factors of the energy metabolism appeared to play an important role in the signalling cascades.

First tissue specific biomarkers could be detected which could be taken to establish a tissue specific screening pattern for anabolic agents.

Tissues, such as the uterus, that are directly influenced by anabolic agents showed very high gene expression regulations. It is supposed that especially these primary sexual organs, e.g. testes or uterus but also blood would be very promising for further biomarker analysis.

The studies showed that AAS provoke very specific signalling pathways and therefore also biomarkers for these substances can be identified. To develop an AAS screening pattern further experiments are necessary to find more biomarkers and to complete the signal cascades of the identified metabolic pathways. Anyhow, first steps toward a new screening method for anabolic agents could be done, that could help to uncover abuses of anabolic agents in the future.

1. INTRODUCTION

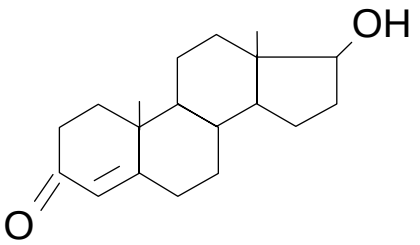
1.1 Anabolic agents

The term “anabolic agents” includes anabolic androgenic steroids (AAS) and other anabolic agents. All these substances have anabolic effects, resulting in the following:

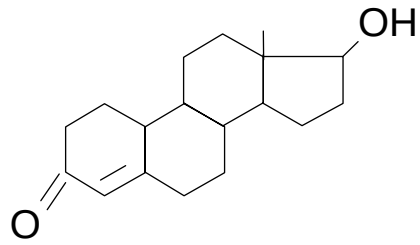
- Increase in muscle mass
- Decrease in total body fat
- Enhancement of protein accretion ⇒ positive nitrogen balance
- Increase in calcium intake of the bone ⇒ positive bone density

Anabolic androgenic steroids

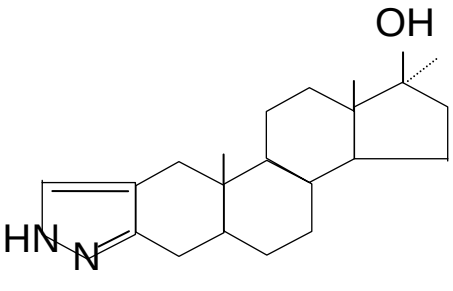
Anabolic androgenic steroids include exogenous and endogenous AAS. Exogenous AAS are not produced by the body naturally and are xenobiotic testosterone analogs, for example stanozolol and metandienone. Endogenous AAS are androgens that are naturally synthesised by the organism, such as testosterone, dihydrotestosterone and 19-nortestosterone (nandrolone). They become prohibited substances when the value found in a sample is unlikely to be consistent with normal endogenous production [1, 2].



Testosterone



19-Nortestosterone



Stanozolol

Figure 1: Testosterone, 19-norstestosterone and the xenobiotic androgen stanozolol.

Testosterone is synthesised from cholesterol and is the most important male sexual hormone. It is mainly produced in the testes and regulates essential functions such as muscle protein metabolism, sexual functions and characteristics, plasma lipid levels and bone metabolism. In females it is also produced in the ovary and the adrenal gland but plays an inferior role. [3].

19-Norstestosterone (nandrolone; 17β-hydroxy-Δ4-estren-3-one) is also an endogenous hormone and compared to 5α-dihydrotestosterone it has a binding affinity of 75% to the androgen receptor. It is known as a performance enhancing substance that increases muscle strength and mass and speeds up recovery. Like testosterone it is hardly orally active and has to be injected into the muscle in form of its ester, called nandrolone decanoate. [4, 5].

Stanozolol (17β-hydroxy-17α-methyl-5α-androst-2-eno[3,2c]pyrazol) is synthesised starting with oxymetholone, which was first done in 1959, and it is active orally. It shows comparable anabolic effects like testosterone [6].

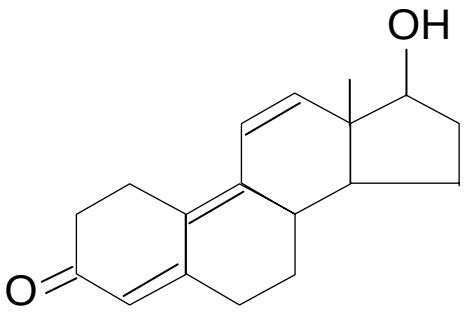
Other anabolic substances

Trenbolone acetate (TBA) is active after hydrolysis to trenbolone-17beta, a 19-norsteroid. It also acts as an anabolic agent and anti-glucocorticoid and provokes the synthesis of myofibrillar proteins in muscle. Trenbolone binds to the androgen receptor with a higher affinity than dihydrotestosterone, to the progestin receptor and even to the glucocorticoid receptor. It is applied as trenbolone acetate in animal husbandry to induce growth [7, 8, 9].

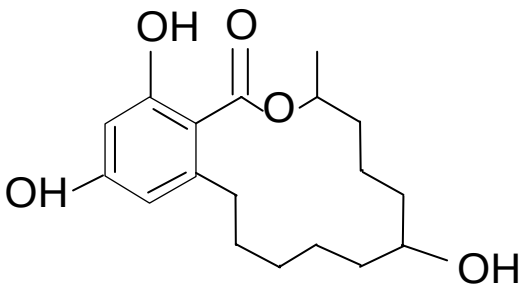
Zeranol has the same effects as estrogen and is derived from the mycotoxin zearalenone. It provokes a higher absorption rate on the regular nutrition level in heifers and improves growth rates in cattle [10, 11].

The xenobiotic gestagen melengestrol acetate (MGA) is orally active and has glucocorticoid and anabolic effects because it promotes growth and increases the endogenous synthesis of estradiol in cyclic females. In the USA and Canada it is licensed for the application in heifers [4].

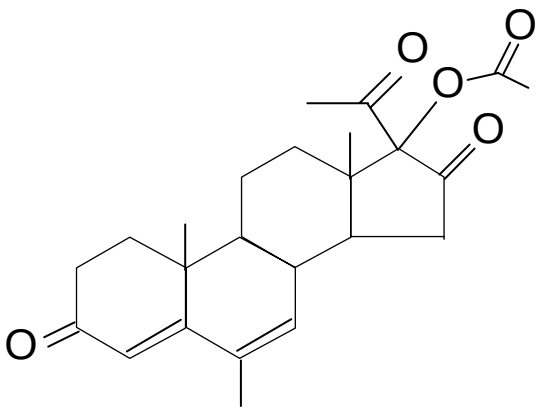
Clenbuterol is a β-agonist. It belongs to the group of β_2-sympathomimetics that are used to relax both the bronchia and the uterus. It is applied in asthma therapy as well as a tokolyticum. Clenbuterol stimulates amino acid retention and inhibits the activity of proteolytic enzymes, such as CTSL and CTSB, which results in an increased muscle growth [12, 13].



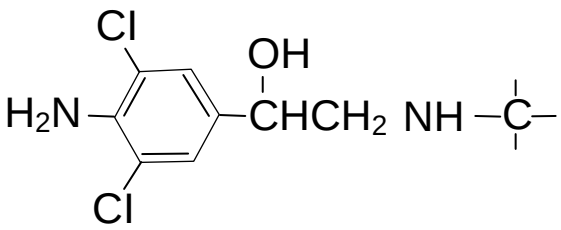
Trenbolone



Zeranol



MGA



Clenbuterol

Figure 2: Other substances with anabolic effects.

1.1.1 Misuse of anabolic agents in sports

After the big doping scandals during the 2007 Tour de France the doping topic has constantly been present in the media and the creeds "fair play" or "give drugs no chance" appear to no longer have any meaning. The abuse of AAS was first reported in the 1950s in samples taken from Soviet weight lifters that captured the substances to gain weight and power. In 1967 the International Olympic Committee (IOC) established a list of prohibited substances that was later adopted by the World Anti Doping Agency (WADA). Today the WADA has composed several anti-doping rules, and the violation of one or more of them is called doping. In most cases this means the intake of forbidden medications that increase performance [14, 15, 16]. The WADA publishes an annual list of prohibited substances that includes all forbidden agents and separates them into functional groups [1]. The following substances and methods are prohibited at all times (in and out of competition):

Prohibited substances:
S1. Anabolic agents
S2. Hormones and related substances
S3. Beta-2 agonists
S4. Agents with anti-estrogenic activity
S5. Diuretics and other masking agents

Prohibited methods:
M1.Enhancement of oxygen transfer
M2. Chemical and physical manipulation
M3. Gene doping

Especially xenobiotic steroids are not only used by competitive athletics. Studies in the USA have shown that more and more pupils take these substances in the hope of obtaining better social prestige and getting a sport scholarship. In Europe the intake of anabolic steroids has become popular with people who have a gym membership, especially with man between 21 and 25 years and whose training goals are to increase muscle mass and gain of strength [17, 18].

1.1.2 Application and misuse of anabolic agents in animal husbandry

The application of anabolic agents in animal husbandry is forbidden since 1988 in the Euoprean Union. In the USA and Canada estradiol-17β, zeranol (estrogens), testosterone, trenbolone (androgens), progesterone, melengestrol acetate (gestagens), ractopamin (β-agonist) and the growth hormone somatotropin are still legally used to induce better growth rate, reduce fat thickness and produce leaner animals with more meat [12,13,19].

The consumption of meat from hormone treated animals may have certain risk for the cunsumers, especially when parts near the point of implantation are eaten. The consequences of an overdose of hormones caused by meat consumption include the disturbance of fertility and the menstruation cycle in women and an increased risk of testicular and prostate cancer in men [20].

To protect the consumer, authorities such as the European Union has established very comprehensive control systems. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) is engaged in collecting and evaluating scientific data on contaminants and residues of veterinary drugs in food. The JECFA has estimated a so-called ADI (Acceptable Daily Intake) that defines the daily and lifelong amount of a substance in food or drinking water without appreciable risk for the consumer. This value is based on pharmacological, microbiological and toxicological studies [4, 21].

Anabolic agent	**ADI** [µg/kg BW per day]
Testosterone	2
Trenbolone	0.02
Zeranol	0.5
MGA	0.03

Tabel 1: Acceptable daily intake (ADI) of anabolic agents used in animal husbandry

European Community Reference Laboratories (CRL) and National Reference Laboratories (NRL) are responsible for residue analysis and the control of adherence to maximum permissible quantities for the European Union. Analytical methods and techniques for residue analysis are established and standardized; therefore the acceptance of additional new techniques as part of enforcement methods is discussed at intervals. New methods can be accepted as long as they are not expensive, are considered to be commonly available and sample preparation is not time consuming [22, 23].

1.1.3 Clinical application of anabolic agents

Anabolic agents are not only used as drugs of abuse to increase performance and strength, but testosterone is also one of the commonly used drugs in androgen replacement therapy. Synthetic androgens are prescribed to treat a variety of male and female disorders, for example hypogonadism, anemias and primary osteoporosis, all resulting from deviant androgen levels [24, 25]. Especially older people and women in the menopause suffer from decreasing hormone levels, which result in sarcopenia and frailty. Already 30% of those over 60 years of age are affected. Sarcopenia, the loss of skeletal muscle mass and strength, results in both men and women in a high incidence of accidental falls and can compromise the quality of life. Chronic, age-related afflictions, e.g. osteoporosis are linked to sarcopenia. A decrease in the production of hormones such as testosterone, estradiol and growth hormone and a general decline in muscle protein turnover as well as neuromuscular alterations, are the major reasons for the appearance of frailty. This results in weakness, impaired mobility and poor endurance - factors that reduce independence and the quality of life [26, 27, 28, 29].
Particularly for women, the therapeutic uses of androgens do not only have beneficial effects, as numerous side effects also exist, e.g. acne, hirsutism, frontal hair thinning and menstrual

disruption [30, 31, 32, 33, 34]. A promising alternative to androgen replacement therapies, for example in case of osteoporosis and frailty, could be the discovery of selective androgen receptor modulators (SARM). SARM are expected to have the ability to mimic the central and peripheral androgenic and anabolic affects of testosterone with lower negative side effects than the natural hormone [35]. Further positive properties of SARM, such as the oral activity, an increase in muscle mass and strength and an increase in fat-free mass, are important factors for its use as a medication [36, 37, 38]. Specific genes could be analysed via mRNA expression in order to identify the influence and effects of SARM in the body and so that the identification of metabolic pathways is possible.

1.2 The idea of biomarkers

The techniques for the identification and characterisation of steroids and their metabolites have changed since the first tests were performed on athletes´ urine in 1976. Until now several methods of detection have been developed, ranging from radio immunoassays to gas chromatography (GC) and mass spectrometry. Today the major metabolites of nearly all AAS are known and several analysis methods exist especially for their detection in urine, blood or hair samples. Various urine tests for detecting testosterone have been developed, for example the calculation of the ratio of testosterone to epitestosterone (T/E), the ketoconazole test and the testosterone glucoronid to luteinising hormone (T/LH) ratio [39, 3, 6].

An important aspect in xenobiotic steroid testing is the question of the origin of the detected substances. Even small amounts of AAS can be detected already in urine samples from people who have ingested meat from clenbuterol or 19-norstestosterone treated animals. But how can a person determine if the steroids analysed have endogenous or exogenous sources? The question remains where to set a limit between illegally taken AAS and the physiological, endogenous production of the hormones [3].

This is where the term “biomarker” becomes important. Several definitions for biomarkers exist, for example the definition from the Food and Drug Administration (FDA): “a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic or pathogenic processes or pharmacological responses to a therapeutic intervention” [40]. Many more definition can be found but all of them try to explain that biomarkers can be used to identify physiological changes in the organism induced by exogenous administered

substances. The identification of such biomarkers in affected tissues could then be used to detect the intake of specific substances, such as anabolic agents.

In theory this would be a new, accurate and easy way to perform drug residue analysis in various species and samples. But at the mRNA expression level a lot of factors exist that could influence these biomarker regulations and that should be kept in mind when developing such a new screening method. The body, human or animal, is exposed to many different environmental factors and especially the analysis of different gene expression regulations can be significantly influenced by factors, such as the condition of the immune system, nutrition or environmental conditions.

Target genes

Steroid hormones can influence the mRNA expression of specific genes by binding to their hormone receptor in the cell. The resulting hormone-receptor complex then can bind to the hormone-responsive-element (HRE), a short nucleotide sequence of DNA that is also called a palindrome or enhancer of transcription. The binding of the hormone-receptor-complex to the HRE activates the transcription of specific genes. The synthesised mRNA is released from the nucleus and is translated into proteins in the cytoplasm [41].

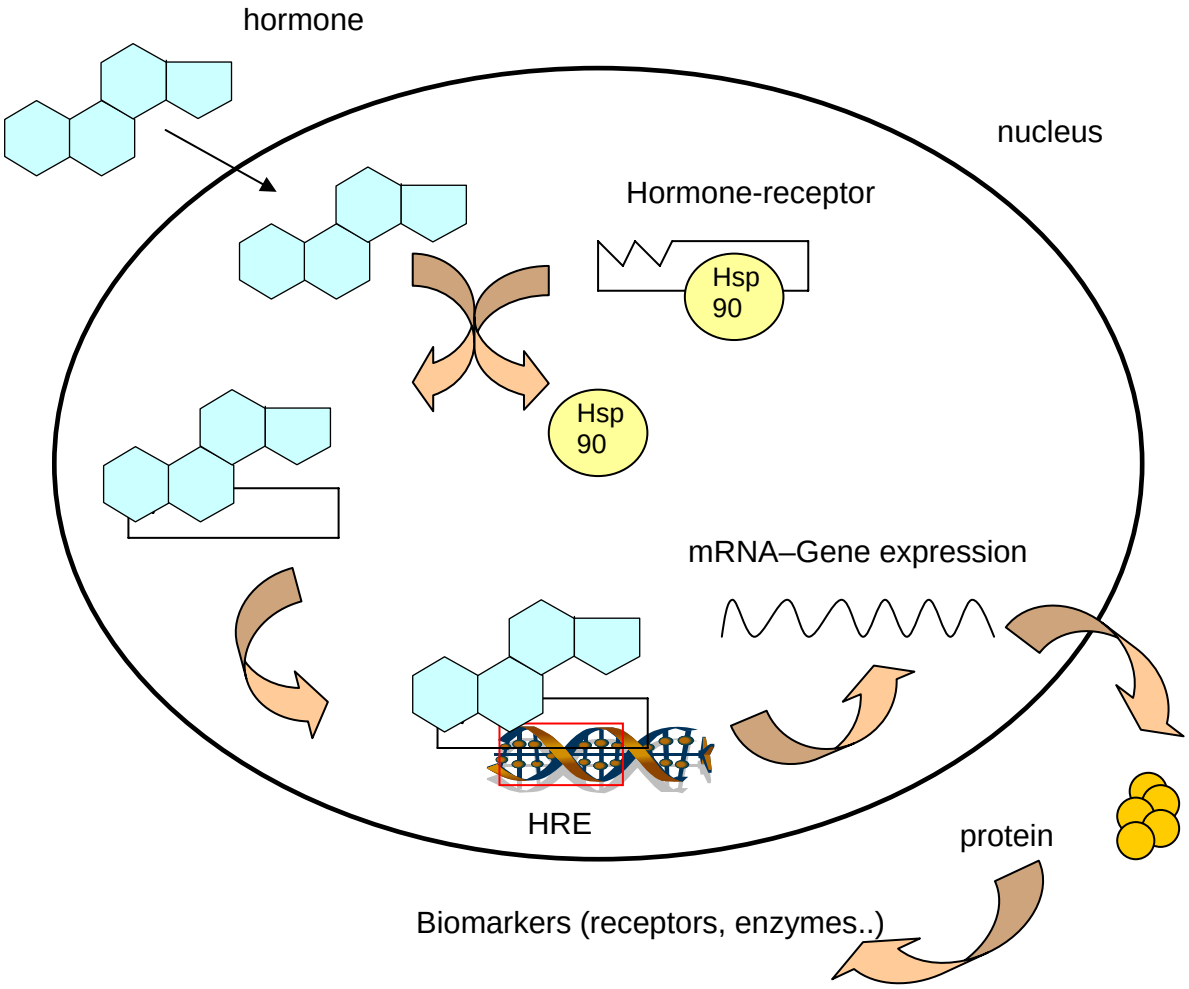


Figure 3: Mechanism of hormone-receptor interaction in the cell. The connection of hormone and hormone-receptor is catalyzed by a heat shock protein (Hsp90). After this the hormone–receptor complex can bind to the hormone responsive element (HRE) which is located on the DNA strand. The synthesised mRNA is released from the nucleus and translated into proteins that code for biomarkers.

Most anabolic agents are hormones that can affect the mRNA expression of specific genes in the cell. These so-called target gene (TG) expressions are on one side tissue dependent, for example in uterus the muscle proteins of smooth muscles. On the other side they can be used as universal factors that are expressed in more than one tissue. After finding target genes that are specifically influenced by anabolic agents the identification of substance specific biomarkers should be possible. Taking these biomarkers, a residue analysis pattern could be developed. Additionally metabolic pathways, influenced by the treatment could be identified and affirm physiological functions.

To measure these specific target gene expressions a method called polymerase chain reaction (PCR) was used. Combining reverse transcriptase (RT) reaction with PCR, it is possible to identify a specific RNA product, what was first done in the early 90s. With the beginning of competitive PCR also the quantification of transcripts from unknown samples could then be

realized. Nowadays PCR has become the most accurate and sensitive method for the detection and quantification of nucleic acids [42]. Therefore this method meets all conditions for biomarker analysis in different tissues.

1.3 Aims

Residue analysis of anabolic agents is an interesting and important issue in food safety and competitive sports. Especially in these areas stringent control systems are necessary because the effects of AAS are often abused.

The objective of the thesis was to proof the possibility to detect AAS treatment via gene expression analysis. Therefore substance specific target genes were selected and analysed in different tissues and species, treated with anabolic agents. Gene expression regulations were measured via qRT-PCR to show up effects on metabolic pathways and to identify possible biomarkers for AAS.

The identified biomarkers could be taken to develop a substance specific gene expression pattern, used for drug residue analysis. Additionally the identified signal cascades could help to understand the influences of newly developed drugs in the organism.

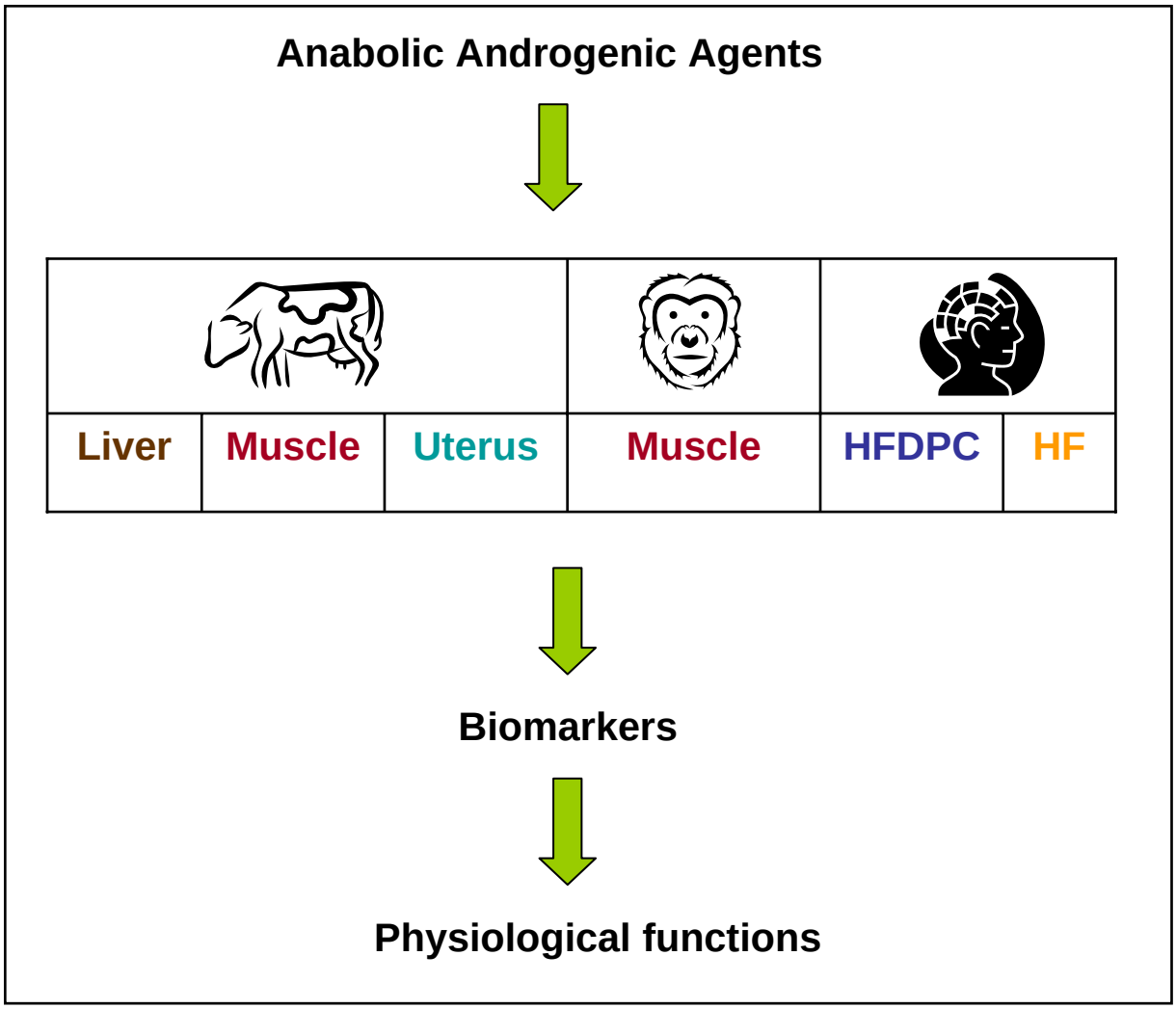


Figure 4: Overview of species and tissues investigated in this thesis with regard of biomarkers and metabolic pathways. Liver, skeletal muscles, uterus, hair follicle dermal papilla cells (HFDPC) and hair follicle (HF) were analysed.

2. MATERIAL AND METHODS

2.1 Studies on bovine tissues

Holstein-Friesian heifers were treated either orally with MGA (0.5mg daily=1fold; Pfizer Animal Health, New York, USA) or with the implant preparations of Finaplix-H® (200mg TBA=1fold; Hoechst Roussel Vet, Sommerville, USA) or Ralgro® (36mg zeranol=1fold; Mallinckrodt Veterinary Inc., Mundelein, USA). According to the manufacturers' instruction, the implants were administered to the middle third of the pinna of the right ear. In every treatment group always 2 heifers were given 1-fold, 3-fold and 10-fold doses of preparation, the control group without any treatment consisted of 2 animals per group. The heifers were aged between 12 to 16 month with a mean weight of 320±57 kg. After 56 days treatment all heifers were slaughtered, tissue samples (liver, *m. splenius*, *m. quadriceps* and uterus) were taken and stored at –80°C until total RNA extraction.

2.2 Studies on primate tissues

This study was done in cooperation with TAP Pharmaceutical Products Inc. (Lake Forest, USA), to identify the influence of a new developed drug, a SARM, on the gene expression in muscle tissues (*m. quadriceps, m. triceps*).

The in-life portions of the study and tissue collection were done by Covance Laboratories GmbH (Münster, Germany). 24 male cynomologous monkeys (*macaca fascicularis*) were separated in four groups, a testosterone group, a SARM1 and SARM10 group and a control group, each consisting of 6 animals. The testosterone group was treated i.m. with 3.0 mg/kg Testostoviron®-depot-250 (testosteronenanthat; Schering, Berlin, Germany) every two weeks; the SARM1 and SARM10 group with 1 mg/kg or 10 mg/kg SARM LGD2941 orally each day, and the control group stayed untreated. All animals were 5-6 years old, skeletally mature and had an average body weight of 6 kg at the start of dosing.

Muscle biopsies of *musculus quadriceps* (*musculus rectus femoris*) and *musculus triceps* were taken at three treatment time points. Predose samples were taken after study start without prior treatment. Further samples were taken at day 16 and day 90 of treatment.

Predose muscle samples (50 mg each) were snap frozen directly in liquid nitrogen, wrapped in aluminium foil and stored at –80°C until RNA extraction. Subsequently muscle samples of

100 mg each were collected on day 16 (by biopsy) and 90 (at the end of the in-life phase), placed in kryotubes before freezing in liquid nitrogen and stored at –80°C until RNA extraction.

2.3 Studies on human tissues

The studies were supported by the Bundesinstitut für Sportwissenschaften (Bonn, Germany) and done in cooperation with the Lehrstuhl für Sport und Gesundheitsförderung, TU Munich and the institute of forensic medicine, TU Munich.
Only very limited numbers of hair follicle samples from treated persons could be taken, therefore a first feasibility study was done in cell culture with human dermal papilla cells, to identify first target genes.
In a second feasibility study a method to take hair follicle samples and extracte total RNA out of them, was tested. Additionally histological stains were prepared from the hair samples to demonstrate if hair follicle cells could be taken by plucking. Also first target genes were measured in hair follicle samples.

2.3.1 Cell culture experiments

Two HFDPC (hair follicle dermal papilla cell) cultures, one from a female and one from a male donor were treated with various concentrations of the anabolic steroids testosterone (4-androsten-17β-ol-3-one), stanozolol (17β-hydroxy-17α-methyl-androstano[3,2-c]pyrazole), nortestosterone (17β-hydroxy-Δ4-estren-3-one) and the β-agonist clenbuterol (clenbuterol hydrochlorid). Samples were taken as indicated below.

Cell culture from female donor
Human HFDPC were ordered from Cell Applications (San Diego, USA). Cells had been cultivated from skin samples of the temple taken during plastic surgery at a 49 year-old, female patient. After establishing a primary cell culture the cells were frozen in serum-free freezing medium and sent cryopreserved (500,000). A ready-to-use HFDPC Medium and Supplement Kit (Cell Applications, San Diego, USA), containing Basal Medium, FCS (fetal calf serum), growth factors and antibiotics were additionally ordered to cultivate the cells. For

sub-cultivation served a kit containing HBSS (HEPES buffered saline solution), trypsin/EDTA solution and neutralizing solution (Cell Applications, San Diego, USA).
The thawed cells were cultivated in collagen coated T-75 flasks (Cell Applications, San Diego, USA) containing 15ml HFDPC medium at 37°C in a humidified atmosphere of 5% CO_2 and sub-cultivated at 85% confluence. Cells were frozen and stored at -80°C after each splitting that was up to the sixth passage. For the experiment cells from third and fourth passage were thawed, given into 12-well plates (ca. $3.4x10^4$/well) and cultivated till 85% confluence. Cells were cultured in triplicates with 0nM (control-treatment), 1nM, 10nM and 100nM treatment groups on each plate. Sampling took place at 0h, 6h, 24h and 48h.
To remove natural containing steroids for all experiments the FCS was stripped by using charcoal, as described by Darbre et al. [43].

Cell culture from male donor

Human HFDPC cells were ordered from Promo Cell (Heidelberg, Germany). Cells had been cultivated from skin samples of the temple taken during plastic surgery at a 40 year-old, male patient. After establishing a primary cell culture the cells were frozen in serum-free freezing medium and sent cryopreserved (500,000 cells). A ready-to-use HFDPC Medium and Supplement Kit (Promo Cell, Heidelberg, Germany), containing Basal Medium, FCS (fetal calf serum), basic FGF and Insulin was additionally ordered to cultivate the cells. Sub-cultivation was done with a kit containing HBSS (HEPES buffered saline solution), trypsin/EDTA solution and neutralizing solution (Promo Cell, Heidelberg, Germany). The experiment was done in the same way as it is described in the cell culture of the female donor but only with 10nM and 100nM treatment concentrations. Sampling took place 0h, 6h, 24h and 72h after treatment.

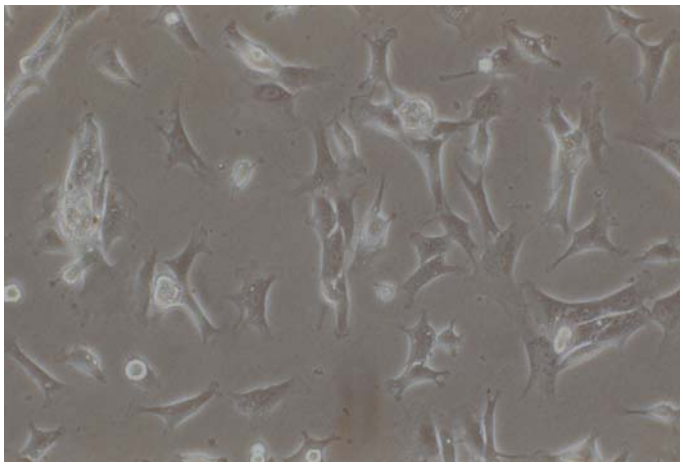


Figure 5: Cultivated hair follicle dermal papilla cells.

2.3.2 Hair follicle experiments

Frontal scalp hair follicle samples were taken from the androgen dependent upper part of the head. In a first study twenty hair follicle samples were taken from women and twenty samples were taken from men to find possible gender specific differences. In a second study three samples from weight lifters taking a mix of anabolic agents (testosterone, TBA) could be taken and three hair samples from untreated man served as control. 5-6 hairs from the upper part of the head were taken by plucking, long hairs were shortened to 1cm and given in 1.5 ml tubes containing lysis buffer (MasterPure RNA Purification Kit, Epicentre Biotechnologies, Madison, USA.). In the first study the tubes were frozen in liquid nitrogen and stored at -80°C, in the second study the RNA extraction was done directly after the plucking.

2.4 RNA extraction, RNA quality

For the RNA extraction different sample amounts had to be taken for the various tissues. For the extraction of the bovine tissues, 200mg of the frozen tissue was taken. In the studies on primate tissues, 50 mg of predose and 100 mg of day 16 and day 90 muscle biopsy samples were used. For the extraction of the HFDPC, 600 µl of TriFast® (peqLab Biotechnologie GmbH, Erlangen, Germany) was directly given in the wells of the cell culture plates. All hair follicle samples that could be gained by plucking were taken for the further extraction.

TriFast® and a standardized protocol from the company were used for the RNA isolation of uterus, muscle and cell culture samples. The principal of this protocol was phenol/chloroform extraction of total RNA. Isolated RNA was diluted in 30µl (liver, uterus and muscle tissue) RNAse free water and stored at -80°C. Because low tissue yield was expected from hair follicle samples a special kit, the MasterPure^{TM} RNA Purification Kit (Epicentre Biotechnologies, Madison, USA.) was used. RNA was extracted following the instruction of the kit what contained proteinase K (50µg/µl) and DNase for RNA purification. RNA was diluted in 10µl TE-Buffer and stored at -80°C.

To quantify the amount of total RNA extracted, optical density (OD) was measured with the photometer (Eppendorf, Hamburg, Germany) or the NanoDrop ND-1000 (peqLab Biotechnologie GmbH, Erlangen, Germany) for each sample. RNA purity was screened taking the $OD_{260/280}$ ratio.

RNA integrity and quality control was performed with the Bioanalyzer 2100 (Agilent Technology, Palo Alto, USA). For sample analysis eukaryotic total RNA Nano Assay (Agilent Technology, Palo Alto, USA) was taken 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 [44, 45].

2.5 Selection of target genes

All target genes were selected if supposed to play an important role in the anabolic pathways of the analysed tissues. Following target genes were chosen by screening the respective literature:

Study in bovine tissues

Liver, muscle and uterus	
Angiogenesis	vascular enothelial growth factors (VEGF120, 164, 188) VEGF receptor (FLK-1) Fibroblast growth factors (FGF2, 7) Angiopoetin (Angpt1, Angpt2) Matrix metalloproteinases (MMP1, MMP2, MMP14) MMP inhibitors (TIMP1, TIMP2)
Apoptosis	Death receptor ligand (FasL) Death receptor (FasR) Caspase 3 Bcl-xl protein (Bcl-xl)
Cell cycle	Cyclin dependent kinase (CDK2) Cyclin A, D1 Myostatin (GDF8)
Endocrine factors	Androgen receptor (AR) Estrogen receptors (ERα, ERβ) Growth hormone receptor (GHR) Prolactin receptor (PRLR) Insulin receptors (IRα, IRβ) Glucocorticoid receptor (GR) Insulin-like growth factor (IGF1) Insulin-like growth factor receptor (IGF1R) Insulin-like growth factor binding protein (IGFBP3)
Energy metabolism	Hexokinase (HK), Lactate-dehydrogenase (LDH) Creatinkinase (CK) Aconitase Acyl-CoA-dehydrogenase (ACADvl) Enoyl-CoA-hydratase (EnoylCoA)
Inflammatory factors	Cycle-oxogenase (Cox2) Interleucin 1 (IL1β) Tumor necrosis factor (TNFα)
Muscle function	α-Actin (ACTA1) Myosin heavy chain (MYHC2x) Myogenic differentiation factor (MyoD)
Oncogenes	Tumor suppressor p53 (p53) Myeloblastosis viral oncogene (v-myb)
Protein metabolism	Cathepsin B, L (CTSB, CTSL) Calpain 3 (CAPN3) Calpastatin (CAST) Tyrosin-amino-transferase (TAT)

Transcription factors	c-fos protein (c-fos) c-jun protein (c-jun)
Reference genes	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) β-Actin (ACTB) Ubiquitin C (UBC)
Others	Cytochrom P1 (CyP1A1) Calreticulin (CALR)

Table 2: List of target genes analysed in bovine tissues [9, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61]

Study in primate tissues

Muscle	
Muscle fibre proteins	Myosin heavy chain (MYHC2x) α-Actin (ACTA1)
Transcription factors	Myogenin Myogenic differentiation factor (MyoD)
Protein metabolism	Cathepsin B, L (CTSB, CTSL) Calpain 3 (CAPN3) Tyrosin-amino-transferase (TAT)
Cell cycle	Cyclin-dependent kinase 2 (CDK2), Cyclin A, D1 Myostatin (GDF8)
Endo- paracrine factors	Androgen receptor (AR), Estrogen receptors (ERα, ERβ), Growth hormone receptor (GHR), Glucocorticoid receptor (GR), Insulin like growth factor receptor (IGF1R), Insulin receptor (IR), Insulin like growth factor binding proteins (IGFBP3, IGFBP4)
Energy metabolism	Hexokinase (HK), Lactate-dehydrogenase (LDH) Creatin kinase (CK) Glucose transporter 4 (GLUT4) Glycogen phosphorylase (PYGM)
Satellite cell biology	Myogenic factors 5, 6 (Myf5, Myf6) Myogenin (MyoG) M-cadherin (CDH15)
Reference genes	β-Actin (ACTB) Cyclophilin A (PPIA), Tyrosin3/tryptophan5-monooxygenase activation protein (YWHAZ) Ubiquitin C (UBC) 18S rRNA

Table 3: List of target genes analysed in primate *m. quadriceps* and *m. triceps* [62, 63, 64, 65, 66, 67, 68, 69 , 70, 71].

Study in human tissues

HFDPC culture and hair follicles	
Apoptosis	Death receptor ligand (FasL) Death receptor (FasR) Caspases 3, 8, 9 B-cell leucemia/lymphoma 2 (Bcl-2) Bcl-xl protein (Bcl-xl)
Growth factors	Tumor necrosis factor (TNFα) Growth hormone receptor (GHR) Insulin-like growth factor (IGF1) Insulin-like growth factor receptor (IGF1R) Insulin-like growth factor binding proteins (IGFBP3, IGFBP5)
Cell cycle	Cyclin D1, E
Enzymes	17β-Hydroxysteroid-dehydrogenase 1,3 (HSD17b1, HSD17b2) Steroid-5α-reductase 1, 2 (SRD5A1, SRD5A2)
Hair metabolism	Fibroblast growth factors (FGF2, FGF7) Fibroblast growth factor receptor (FGF1R, FGF2R) Hepatocyte growth factor (HGF) Neurotrophin (NT5) Interleukin 1 beta (IL1β)
Steroid receptors	Androgen receptor (AR) Estrogen receptors (ERα, ERβ) Glucocorticoid receptor (GR)
Reference genes	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) β-Actin (ACTB) Ubiquitin C (UBC).

Tabel 4: List of analysed target genes in the HFDPC cell culture and hair follicle experiments [72, 73, 74, 75].

Full names of the target genes can be found under the chapter "Abbreviations".

2.6 Primer design and agarose gel electrophoresis

All primers were designed using published nucleic acid sequences of Ensembl Genom browser [76] and NCBI [77]. Primer design and optimization was done with primer design program primer 3 [78] with regard to primer dimer formation, self-priming formation and primer annealing temperature at 60°C. Designed primers were ordered and synthesized at

MWG Biotech (Ebersberg, Germany). Primer testing was performed with three samples and a negative control (RNAse free water) for each primer set. With the PCR products an agarose gel electrophoresis was done to check the primer length and absence of primer dimers.
High resolution agarose gel and electrophoresis was conducted at 90 V for 30 minutes. Agarose gel was then photographed under UV-excitation to enable analysis of fragment size, band intensity and integrity.

2.7 Real-time qRT-PCR

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 manufacture. With the kit the master mix was prepared as follows: For one sample was calculated 5µl 2x SYBR Green Reaction Mix, 0.5µl forward primer (10 pmol/µl), 0.5µl reverse primer (10 pmol/µl) and 0.2µl SYBR Green One-Step Enzyme Mix. 6.2µl of the master mix was filled in the special 100 µl tubes and 1 ng/µl total RNA in 3.8 µl volume was added. The following uniform RT-PCR protocol was used for all genes:

One Step qRT-PCR Protocol, Invitrogen		
Step	**Temperature**	**Time**
I(hold, RT)	55°C	10 min
II(denaturation)	95°C	5 min
III(cycling)	40 cycles	
1.(denaturation)	95°C	15 sec
2.(annealing)	60°C	30 sec
3.(elongation)	68°C	20 sec
IV(hold2)	40°C	1 min
V	Melting Curve Analysis	60°C-95°C; 0.5sec

Tabel 5: One-step qRT-PCR protocol for used real-time PCR instruments.

All experiments were done with the Rotor-Gene instruments from Corbett (Corbett Life Science, Sydney, Australia). Studies on bovine tissues were done with the Rotor-Gene 3000, studies on primate and human tissues with the Rotor-Gene 6000.
Crossing Points (Ct) and melting curves were acquired by using the *"Quantitation"* (cell culture experiment, hair follicle experiment), *"Comparative quantification"* (muscle

experiment, uterus experiment) and *"Melting curve"* program of the Rotor-Gene 3000 and 6000 analysis software. All samples were baseline corrected and threshold was set manually in the cell culture and hair follicle experiment, using same threshold levels for one gene in all samples of an experiment.
Only genes with clear and single melting peaks were taken for further data analysis, samples with irregular melting peaks were excluded.

2.8 Data Analysis and Statistics

Data were processed applying relative quantification method comparable to the ΔΔCt-method ($2^{\Delta\Delta Ct}$), also abbreviated as ddCt [79, 80, 81]. Expression changes are shown as relative up- or down-regulation compared to the three internal reference genes. For normalization of target gene expression the arithmetic mean (AM) of the non-regulated reference genes were taken and the reference gene index (RGI) was calculated.

Reference genes							
Human tissues				**Primate tissues**	**Bovine tissues**		
Cell culture experiments		**Hair follicle experiments**		**Muscles**	**Liver**	**Muscles**	**Uterus**
1	**2**	**1**	**2**				
ACTB	ACTB	s.r.	ACTB	ACTB	ACTB	s.r.	s.r.
UBC	UBC	UBC	UBC	PPIA	UBC	UBC	UBC
s.r.	GAPDH	GPDH	s.r.	YWHAZ	GAPDH	ACTB	GAPDH

Tabel 6: List of reference genes, taken for relative quantification in the different studies.
s.r. = significantly regulated.

In the first step of the ΔΔCt-method the crossing point of the AM of the target genes was subtracted from the crossing point of the reference gene index. From the so calculated ΔCt the mean value of the repeat determination was taken to get the ΔCt of the treatment. From this $\Delta Ct_{(treatment)}$ the mean value of $\Delta Ct_{(control)}$ of the non treated control was subtracted. The result was the ΔΔCt value. In this calculation model the optimal PCR efficiency, representing a DNA doubling after each cycle (efficiency = 2), is assumed. Therefore the expression ratio of a target gene compared to the control treatment of the analysis expected as $2^{-\Delta\Delta Ct}$.

$$\Delta Ct = Ct_{(TG)} - Ct_{(RGI)}$$

$\Delta Ct_{(treatment)}$ = mean ΔCt value of repeat determination of treated animals

$\Delta Ct_{(control)}$ = mean ΔCt value of control animals or control treatment

$$\Delta\Delta Ct = \Delta Ct_{(treatment)} - \Delta Ct_{(control)}$$

$$\text{Ratio} = 2^{-\Delta\Delta Ct}$$

In the studies on bovine and primate tissues the expression ratio $2^{-\Delta\Delta Ct}$ was calculated for all significantly regulated target genes. If the expression ratio was < 1 the expression of the gene was down-regulated, if the expression ratio was > 1 the gene was up-regulated. In the bovine studies the regression of $\Delta\Delta$Ct over treatment time was calculated in a linear regression model to show the gene regulation over time. Additionally, in the studies on primate tissues statistical descriptions of the expression data as well as statistical tests were produced with SAS v. 9.1.3 for Windows. With box-whisker plots the visual screening of regulated genes was possible.

In the cell culture and hair follicle experiments $\Delta\Delta$Ct was calculated, regulations over treatment time were evaluated using a 2-Way-Anova with SigmaStat 3.0 Software (SPSS, Munich, Germany).

All $\Delta\Delta$Ct calculations were done in Excel (Microsoft, USA) by using the t-test. All data were illustrated by means±standard deviation (S.D.), significance minimum was $p<0.05$.

3. RESULTS AND DISCUSSION

All feasibility studies were done using similar methods and instruments, to make results comparable.

3.1 Studies on bovine tissues

RNA concentration and RNA integrity

Total RNA concentration from liver samples was in mean±S.D. 2084.9±765.1 ng/µl, in the muscles 1244.3±908.1 ng/µl and in the uterus 3320.1±212.9 ng/µl. In the liver, muscles and uterus the mean RIN were 7.7, 7.6, and 7.3. All tissue samples were taken in 1998, so they kept frozen at -80°C for seven years until extraction. Anyhow, the RIN showed quite good results and so samples could be taken for further qRT-PCR analysis.

Primer design

Primer pairs of 57 genes were used for qRT-PCR analysis to get single peaks and uniform melting curves, as well as a specific single band in agarose gel electrophoresis. All primer pairs listed below showed satisfactory results in PCR analysis and could be used for further calculations.

GROUP	INDEX	SEQUENCE 5´→ 3´	BP	AT (°C)	ACC.NR.
angiogenesis	Angpt 1 for	TTC CTC GCT GCT ATT CTG ACT	194	58	*
	Angpt 1 rev	TGA CAG CAC TCT CAT GCT GTC			
	Angpt2 for	AAT TCA GT CTC CAA AAG CAG C	234	58	*
	Angpt2 rev	TCC ACC CGT TTC CAT GTC			
	FGF2 for	AGC CTT GCA ACT CTG CTT GT	210	60	*
	FGF2 rev	CGA ATT CAG ATC CCT CCT GA			
	FGF7 for	CTG CCA AGT TTG CTC TAC AG	294	60	*
	FGF7 rev	TCC AAC TGC CAG GGT CCT GAT			
	FLK-1 for	GCT TCT ACC AGG ACA CTG ACA T	144	62	*
	FLK-1 rev	AAC ACG GAA TCA CCA CCA CAG TT			
	MMP1 for	GAG GAG ACG CTC ATT TTG ATG	235	60	NM174112
	MMP1 for	ACT GGC TGA GTG GGA TTT TG			
	MMP2 for	CCC AGA CAG TGG ATG ATG C	237	60	NM174745
	MMP2 rev	TTG TCC TTC TCC CAG GGT C			
	MMP14 for	ACT TGG AAG GGG GAC ACC	232	60	*
	MMP14 for	AGG GGG CAT CTT AGT GGC			
	TIMP1 for	CAT CTA CAC CCC TGC CAT G	231	60	NM174471
	TIMP1 rev	CAG GGG ATG GAT GAG CAG			

	TIMP2 for	GGG TCT CGC TGG ACA TTG	255	59	NM174472
	TIMP2 rev	TTG ATG TTC TTC TCC GTG ACC			
	VEGF all for	CCG TCC CAT TGA GAC CCT G			*
	VEGF120 rev	CGG CTT GTC ACA ATT TTT CTT GTC	280	59	*
	VEGF164 rev	GCC CAC AGG GAT TTT CTT GC	278	59	*
	VEGF188 rev	TGC CCC TTT CCC TTT CCT C	296	60	*
	VEGF for	GGT GGA CAT CTT CCA GGA GTA	177	60	NM174216
	VEGF rev	CTA TGT GCT GGC TTT GGT GAG			
apoptosis	bcl-xl for	GGC ATT CAG CGA CCT GAC	203	60	AF245487
	bcl-xl rev	CCA TCC AAG TTG CGA TCC			
	Caspase 3 for	GCA ACG TTT CTA AAG AAG ACC ATA G	64	60	NM174260
	Caspase 3 rev	CCA TGG CTT AGA AGC ACA CAA ATA A			
	Fas-L for	CAT CTT TGG AGA AGC AAA TAG	205	60	AB035802
	Fas-L rev	GGA ATA CAC AAA ATA CAG CCC			
cell cycle	CDK2 for	CTC ATC GAG TCC TGC ACC G	183	60	BT020790
	CDK2 rev	GTA GTA TTT GCA GCC CAG AAG G			
	Cyclin A for	GCT ATC CTC GTG GAC TGG TTA	159	60	X68321
	Cyclin A rev	AAG CAT AGC AGC AGT GCC CA			
	Cyclin D1 for	GAA GAT GAA GGA GAC CAT CCC	190	60	*
	Cyclin D1 rev	TTG TTC TCC TCT GCC ACT GG			
	Myostatin for	GCC TGT CAA GAC TCC TGC GA	173	60	AY160688
	Myostatin rev	TGT TTG AGC CAG TTC TGC AAC AC			
endocrine factors	AR for	CCT GGT TTT CAA TGA GTA ACC GCA TG	172	60	AY862875
	AR rev	TTG ATT TTT CAG CCC ATC CAC TGG A			
	ERα for	AGG GAA GCT CCT ATT TGC TCC	234	60	AF177936
	ERα rev	CGG TGG ATG TGG TCC TTC TCT			
	ERβ for	GAG ATA TTC TTT GTG TTG GAG TTT	242	60	*
	ERβ rev	CTT CGT GGA GCT CAG CCT GT			
	GHR for	CCA GTT TCC ATG GTT CTT AAT TAT	136	60	NM176608
	GHR rev	TTC CTT TAA TCT TTG GAA CTG G			
	GR for	TTC GAA GAA AAA ACT GCC CAG C	190	60	AY238475
	GR rev	CAG TGT TGG GGT GAG TTG TG			
	IGF1 for	CCA AAG GCC AGA CCT ACT TG	180	60	X15726
	IGF1 rev	TCC TCA GAT CAC AGC TCC GA			
	IGF1R for	TTA AAA TGG CCA GAA CCT GAG	314	60	X54980
	IGF1R rev	ATT ATA ACC AAG CCT CCC AC			
	IGFBP3 for	ACA GAC ACC CAG AAC TTC TCC T	202	60	NM174556
	IGFBP3 rev	AGA AAC CCC GCT TCC TGC C			
	IRα for	TCC TCA AGG AGC TGG AGG AGT	89	60	AJ320235
	IRα rev	TTT CCT CGA AGG CCT GGG GAT			
	IRβ for	TCC TCA AGG AGC TGG AGG AGT	111	60	AJ320235
	IRβ rev	TAG CGT CCT CGG CAA CAG G			
	PRLR for	AAG GAA GGA GAA ACA CTC ATC CA	203	60	L02549
	PRLR rev	AGG TTT GCA GGA GGC TCT G			
energy metabolism	ACADvl for	ACT TTG ACG GAG TAC GGG TG	198	60	U30817
	ACADvl rev	CAA AGT TGT GAA TTT TCT CCC C			
	Aconitase for	CAT CCG AGT TGG TCT GAT TG	188	60	NM173977
	Aconitase rev	ACA TCC CTC AGG ATC TGT G			
	CK for	ATG ACA GAG CAG GAG CAG CA	183	60	BT021173
	CK rev	ATG GAG ATG ACT CGG AGG TG			
	EnoylCoA for	GCT GCT GTC AAT GGC TAT GC	202	60	BT021569

	EnoylCoA rev	ACC AGT GAG GAC CAT CTC CA			
	HK for	CAA GAC GCA CCC ACA GTA TCC	211	60	NM001012668
	HK rev	TCA CCT CCA GCA GCA TTT CCT T			
	LDH for	GTG GCT TGG AAG ATA AGT GG	155	60	NM174099
	LDH rev	ACT AGA GTC ACC ATG CTC C			
inflammatory factors	Cox2 for	CCT GAT GAC TGC CCA ACA C	162	60	AF004944
	Cox2 rev	AAA TTG ATG GGT GAA GTG CTG G			
	IL1β for	TTC TCT CCA GCC AAC CTT CAT T	198	60	M37211
	IL1β rev	ATC TGC AGC TGG ATG TTT CCA T			
	TNFα for	TAA CAA GCC GGT AGC CCA CG	256	60	AF011926
	TNFα rev	GCA AGG GCT CTT GAT GGC AGA			
muscle function	ACTA1 for	TAT TGT GCT CGA CTC CGG CGA	160	60	NM174225
	ACTA1 rev	GTC ACG AAG GAG TAG CCA CG			
	MYHC2x for	GAT CAA TGC TGA GCT GAC GG	173	60	AB059399
	MYHC2x rev	CAA TGG TTT CAT CCA GAC CTG C			
oncogenes	p53 for	GAC TTC TCT CGG CGC CTA CC	191	60	X81704
	p53 rev	TGG TGC ACT CAG AGT CGA TC			
	v-myb for	TCA CGT CCC ATA TCC TGT AGC	170	60	NM175050
	v-myb rev	CCT GTC CTT TGA GTT CGT TCT CA			
protein metabolism	CAPN3 for	GAC TGG AGC TAT GTG GAC AAG	179	60	NM174260
	CAPN3 rev	GTC CAA GTC TGA AGC TTG TCG			
	CAST for	GAT CAG AAG TGC TGC TCC A	206	60	NM174003
	CAST rev	GGA CTG TTT CCT CAT CTT ACC			
	CTSB for	GAT CTG CAT CCA CAG CAA	192	60	NM174031
	CTSB for	ATG GAG TAC GGT CTG CAA CC			
	CTSL for	CAC TGG TGC TCT TGA AGG ACA	183	60	NM174032
	CTSL rev	TAA GAT TCC TCT GAG TCC AGG C			
	TAT for	ACC CTT GTG GGT CAG TGT TC	167	60	BT021798
	TAT rev	ACA GGA TGG GGA CTT TGC TG			
transcription factors	c-fos for	GCT CCA GGC GGA GAC AGA	302	60	AY069515
	c-fos rev	AGG GTG AAG GCC TCC TCA GA			
	c-jun for	TCA ACG CCT CGT TCC TCC	278	60	AF069514
	c-jun rev	CTC ATC TGT CAC GTT CTT GGG GCA			
reference genes	ACTB for	AAC TCC ATC ATG AAG TGT GAC	202	60	AY141970
	ACTB rev	GAT CCA CAT CTG CTG GAA GG			
	GAPDH for	GTC TTC ACT ACC ATG GAG AAG G	197	60	U85042
	GAPDH rev	TCA TGG ATG ACC TTG GCC CAG			
	UBC for	AGA TCC AGG ATA AGG GAA GGC AT	198	60	Z18245
	UBC rev	GCT CCA CCT CCA GGG TGA T			
others	CyP1A1 for	CCG ACC TCT ACA GCT TCA C	181	60	AB060696
	CyP1A1 rev	GCC TCC TTG TTC ACA TGC TC			
	CALR for	TTG ACA ACA GCC AGG TGG AG	192	60	NM007591
	CALR rev	CAG GTT TCT TAG CAT CAG GGT C			

Table 7: List of designed primer pairs for all bovine tissues. Length of the amplicons (BP) and annealing temperatures (AT) are listed. Some primer pairs were taken from extern references (*), so the accession number was not available.

qRT-PCR and gene expression regulations in liver, muscle and uterus

Tissue specific biomarkers were analysed to show first metabolic effects in the different tissues and treatment dependent regulations.

Liver		M. splenius		M. quadriceps		Uterus	
TG	**TM**	**TG**	**TM**	**TG**	**TM**	**TG**	**TM**
EnoylCoA ↑↑	TBA	MYHC2x ↓	TBA	MYHC2x ↓	TBA	ACTA1 ↓	Z
ACADvl ↑↑	TBA	ACTA1 ↓	MGA, TBA	CTSL ↑	TBA, Z	CTSL ↑↑	TBA
FasL ↑↑	TBA	CDK2 ↑	MGA	Cyclin A ↑	MGA	MMP2 ↓	MGA
Bcl-xl ↑↑	TBA	Cyclin A ↑	Z	AR ↓	TBA	TIMP2 ↑↑	MGA
IGF1 ↑↑	Z	GDF8 ↓	TBA	IGF1 ↑	Z	Angpt2 ↓↓	TBA
IRα ↑	TBA	IGF1 ↑	Z	GRα ↑, ↓	MGA, TBA	AR ↑	TBA
IRβ ↑	MGA	IGFBP3 ↑	MGA	LDH ↓↓	TBA	ERβ ↓↓	TBA
PRLR ↑↑	MGA	ERα ↑, ↓	MGA, Z			IGFBP3 ↑↑	TBA
GR ↑↑	Z	GRα ↑, ↓	MGA, TBA			HK ↓↓	Z
GHR ↑↑	MGA, TBA	HK ↑	TBA			Aconitase ↑	MGA, TBA
IL1 β ↑	TBA	LDH ↓	TBA			FasL ↑	MGA, TBA
Cox2 ↓	MGA					Bcl-xl ↓	Z
CAST ↑	MGA					Caspase 3 ↑	TBA
CTSB ↑↑	TBA						
CTSL ↑↑	MGA						
p53 ↓	MGA						
v-myb ↑↑	MGA, TBA						
c-fos ↑	MGA						

Tabel 8: Tissue depentent biomarkers in bovine samples.The arrow shows the up or down-regulation of the target gene (TG). P-value<0.05 is shown by one arrow, p-value<0.01 is shown by two arrows. Treatments (TM) were melengestrol acetate (MGA), trenbolone acetate (TBA) and zeranol (Z).

Metabolic pathways in the liver

Especially genes of the endocrine (IGF1, IRα, IRβ, GR, PRLR, GHR) adipolysis (ACADvl, EnoylCoA) and oncogene systems (v-myb, p53) were regulated and showed first auspicious effects.

The adipolysis factors ACADvl and EnoylCoA showed significant up-regulations (71%; 76%) in the liver under TBA treatment. One central reaction during the fatty acid oxidation is the ACADvl induced oxidation of Acyl-CoA with the formation of Enoyl-CoA followed by another essential reaction, which is the EnoylCoA enzyme induced hydratisation with the formation of hydroxyacyl-CoA. At the end of the fatty acid oxidation Acetyl-CoA is produced which feeds into the citric acid cycle of energy-yielding reactions in the mitochondrial matrix

or is used to form keto acids [82]. To show next metabolic pathways of Acetyl-CoA further factors of the citric acid cycle or the ketose pathways need to be analysed.

Metabolic pathways in the muscles

In both muscles very similar gene regulations could be identified. Muscle proteins (MYHC2x, ACTA1), enzymes of the energy metabolism (HK, LDH) and cell cycle factors (Cyclin A, GDF8, CDK2) indicated a possible influence of AAS on theses pathways.
In *m. splenius* factors of energy metabolism, such as HK were up-regulated (62%), LDH was down-regulated (51%) under TBA treatment. HK catalyse the formation of glucose to glucose-6-phsophate. The endproduct of the cascade is pyruvat that can be convertet to lactate by LDH. This last step is called anaerobic way of glycolysis [39]. AAS seem to induce the aerobic and inhibit the anaerobic way of glycolysis but again further enzymes of the cascade have to be tested to confirm the consumption.

Metabolic pathways in the uterus

In this tissue the angiogenesis factors (MMP2, TIMP2, Angpt2), the apoptosis factors (FasL, Caspase 3, Bcl-xl) and the steroid receptors (AR, ERβ) seem to be affected pathways.
As primary sexual organ, the uterus showed the highest gene expression regulations. Especially the apoptosis factors showed related regulations. FasL and Caspase 3 were significantly up-regulated (263%; 151%) under TBA treatment. The extrinsic way of apoptosis is induced by the death receptor ligand FasL which binds to death receptor FasR. The complex can transform procaspase 8 to Caspase 8 which then influences Caspase 3. Caspase 3 induces apoptosis [83]. It is supposed that AAS influence this way of apoptosis.

First tissue and treatment specific biomarkers could be identified. Additionally first modifications on metabolic pathways are supposed to be induced by AAS. In the liver the various gene regulations were expected because of its role as multi functional accumulator. The primary sexual organs, the uterus, turned out to be very promising for gene expression regulations.

3.2 Studies on primate tissues

RNA concentration and RNA integrity

The mean±S.D. total RNA concentration of the predose samples was 947.9±387.1 ng/µl for the *m. quadriceps* and 515.8±209.5 ng/µl for the *m. triceps*. The day 16 samples showed a total RNA concentration of 1384.9±455.8 ng/µl for the *m. quadriceps* and 1141.8±565.9 ng/µl for the *m. triceps*. 1573.7±375.1 ng/µl total RNA for the *m. quadriceps* and 1563.3±396.9 ng/µl for the *m. triceps* samples in day 90 could be measured.

The first measurements of predose samples showed a very high 5S RNA peak for all samples which indicates partly degraded total RNA and lower RNA quality. Because predose biopsy samples were directly frozen in liquid nitrogen without protection of the sample, it is possible that the RNA was damaged and resulted in a partly degraded total RNA with a high 5S RNA peak. To address this problem the sensitivity of the 5S region was augmented in the algorithm of the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA) with the result that the significant 18S and 28S regions could be identified.

The mean RIN value was 6.5 for the *m. quadriceps* and 6.9 for the *m. triceps* predose samples, representing a total RNA of average quality.

At the second sampling on day 16, the biopsy tissues were placed into kryotubes before freezing in liquid nitrogen. In RNA integrity analysis all samples showed a lower 5S region than the predose samples, but for standardization the 5S peak sensitivity was also augmented. The *m. quadriceps* samples had a mean RIN of 7.1, the *m. triceps* of 7.6.

For the day 90 samples the standardization the 5S peak sensitivity was augmented because the 5S peak was still dominant. *M. quadriceps* samples had a mean RIN of 6.9, *m. triceps* samples of 7.6. For each time point, the *m. triceps* samples showed better RNA quality with less variation than the *m. quadriceps* samples.

The low RNA quality of predose samples clearly showed that muscle biopsy samples should not be stored directly in liquid nitrogen. Because of the small surface of the samples it seems that the liquid nitrogen totally destroyed the muscle tissue cells and the included RNA. The samples frozen in kryotubes (days 16 and 90) appeared to not have this kind of freezer burn because more total RNA could be isolated and was still intact in these samples.

However, muscle tissue samples from euthanized animals (day 90) resulted in better quality RNA than the biopsy samples from day 16. Most of the biopsy samples were coated with

blood and included connective tissue, factors that influence the pureness of muscle cell RNA. The samples collected at day 90 did not appear to include blood or connective tissue.

Primer design

Again only primers that showed a specific PCR product in PCR analysis were taken for further statistical analysis.

GROUP	IDENTITY	SEQUENCE 5´→ 3´	BP	AT (°C)	ACC.NR.
muscle fibre proteins	ACTA1 for	CAT GGT CGG TAT GGG TCA GAA	154	60	NM001100
	ACTA1 rev	CGC GAA GCT CGT TGT AGA AG			
	MYHC2x for	GCA CAT CCA GAG CAG AGA AGA A	229	60	NM017534
	MYHC2x rev	GAC AGT GAC ACA GAA GAG ACC T			
transcription factors	Myogenin for	ATC ATC TGC TCA CGG CTG AC	108	60	NM002479
	Myogenin rev	TTT CAT CTG GGA AGG CCA CAG A			
protein metabolism	CTSB for	ACAATTCCTACAGCGTCTCC	299	60	L16510
	CTSB rev	CACCACTTCTGATTCGATTCC			
	CTSL for	ACATCCCTAAGCAGGAGAAGGC	159	60	M20496
	CTSL rev	CCAGCACACCATGATCCATGTC			
	CAPN3 for	TCCAGTTCGTCTGGAAGAGACC	273	60	AY902237
	CAPN3 rev	TGTACGTTGGCAGGCAGTCATC			
cell cycle regulation	CyclinD for	AAC AAG CTC AAG TGG AAC CTG G	170	60	NM053056
	CyclinD rev	CAT GGA GGG CGG ATT GGA AAT			
	Myostatin for	AAC TTG ACA TGA ACC CAG GCA C	177	60	NM005259
	Myostatin rev	ACG GAT TCA GCC CAT CTT CTC			
endo-paracrine factors	AR for	TTG TCC ATC TTG TCG TCT TCG G	237	60	L29496
	AR rev	TGT CCA GCA CAC ACT ACA CC			
	ERβ for	ATG CTC ACT TCT GCG CTG TC	219	60	NM001437
	ERβ rev	CAC ACT TCA CCA TTC CCA CTT C			
	GHR for	ATC CAC CCA TTG CCC TCA AC	246	60	NM00163
	GHR rev	ATC TCA CAC GCA CTT CAT ATT CC			
	IGF1R for	CAT TTC ACC TCC ACC ACC AC	151	60	NM000875
	IGF1R rev	AGG CAT CCT GCC CAT CAT AC			
	IGFBP3 for	CAC AGA TAC CCA GAA CTT CTC C	227	60	M35878

	IGFBP3 rev	CCA TAC TTA TCC ACA CAC CAG C			
	IGFBP4 for	CAA CTT CCA CCC CAA GCA GT	130	60	NM001552
	IGFBP4 rev	CTG GTG GCA GTC CAG CTC			
	IR for	TCC AGA CAG ATG CCA CCA AC	280	60	NM000208
	IR rev	ATC CAG CTC GAA CAG CTC AC			
	GR for	TTC TGC GTC TTC ACC CTC AC	159	60	AH002750
	GR rev	CTG TCT CTC CCA TAT ACA GTC C			
energy metabolism	HK for	CGC ATC TGC TTG CCT ACT TC	229	60	NM000189
	HK rev	AAC TCT CCG TGT TCT GTC CC			
	LDH for	TTC AGC CCG ATT CCG TTA CC	226	60	NM005566
	LDH rev	CAC CTC ATA AGC ACT CTC AAC C			
	CK for	GAC ATC GTC TAC AGT GAA GCC	157	60	NM001824
	CK rev	AGT TCA AGC TGA ATT TAC AAG CCT G			
	PYGM for	ACA TCA ACC CCA ACT CAC TCA C	160	60	AF066859
	PYGM rev	GCC TTC CCT CCA ATC ATC AC			
satellite cell biology	Myf5 for	TGA GAG AGC AGG TGG AGA AC	290	60	NM005593
	Myf5 rev	GGC AAC TGG AGA GAG AGA AG			
	Myf6 for	GCC AAG TGT TTC CGA TCA TTC C	166	60	NM002469
	Myf6 rev	ACT TCT CCA CCA CTT CCT CC			
reference genes	ACTB for	TATAA Biocenter		60	*
	ACTB rev	TATAA Biocenter			*
	PPIA for	TATAA Biocenter		60	*
	PPIA rev	TATAA Biocenter			*
	YWHAZ for	GCA ACC AAC ACA TCC TAT CAG AC	243	60	NM145690
	YWHAZ rev	TTC TCC TGC TTC AGC TTC GTC			

Table 9: List of designed primer pairs for both primate muscles. Length of the amplicons (BP) and annealing temperatures (AT) are listed. Some primers (*) were taken from the human endogenous control panel kit (TATAA Biocenter, Gothenburg, Sweden), therefore primer sequence and accession number was not available.

It was not possible to design perfect and working primer pairs for all target genes. The reason was that few gene sequences of *macaca fascicularis* are available in public gene banks so the gene sequences of *homo sapiens* were used for primer pair design. Because the genome of cynomolgous monkey does not correlate hundred per cent with the genome of *homo sapiens*, the chance of designing non-annealing primer pairs is still high.

Results and discussion of qRT-PCR and gene expression regulations in the muscle

Measured target genes in muscle biopsy samples:

	M. quadriceps			**M. triceps**		
	Predose	**Day 16**	**Day 90**	**Predose**	**Day 16**	**Day 90**
ACTA1	✓	✓	✓	✓	✓	✓
MYHC2x	✓	✓	✓	✓	✓	✓
MyoG	✓	✓	✓	✓	✓	✓
CTSL	✓	✓	✓	✓	✓	✓
CTSB	*no result*	✓	✓	✓	✓	✓
CAPN3	✓	✓	✓	✓	✓	✓
Cyclin D1	✓	✓	✓	✓	✓	✓
Myostatin	✓	✓	✓	✓	✓	✓
AR	*no result*	✓	✓	✓	✓	*no result*
ERα	excluded					
ERβ	✓	✓	*no result*	✓	✓	*no result*
GHR	✓	✓	*no result*	excluded		
GR	✓	✓	✓	✓	✓	✓
IGF1R	✓	✓	*no result*	✓	*no result*	*no result*
IGFBP4	✓	✓	✓	✓	✓	✓
IGFBP3	✓	✓	✓	✓	✓	✓
IR	✓	✓	✓	✓	✓	✓
HK	*no result*	✓	✓	*no result*	✓	*no result*
LDH	✓	*no result*	*no result*	✓	✓	*no result*
CK	✓	✓	✓	✓	✓	✓
Myf5	✓	✓	*no result*	excluded		
Myf6	✓	✓	✓	✓	✓	*no result*
CDH15	excluded					
PYGM	✓	✓	✓	excluded		

Table 10: Measured target genes in *m. quadriceps* and *m. triceps* biopsies of *macaca fascicularis*. ✓means TG with specific PCR products. No results indicate an unsatisfactory melting curve. Genes that did not show specific products in all three time points were excluded.

In *m. quadriceps* significant differences between the control and the treatments could be identified after 90 day treatment, including the CTSL (p-value=0.0138), CAPN3 (p-value=0.0625) and IGFBP3 (p-value=0.0280) (Figure 1, 2, 3). There were no significantly regulated genes found in *m. triceps*. Possible effect of outliers was investigated and could not be excluded. Only the three significantly regulated genes on the time point 90 days in *m. quadriceps* are hence discussed further. Surprisingly the expression ratio deviated from the

value one in the control group, showing thus endogenously induced up-regulation throughout the growth period of the animals. This conclusion is supported by the fact that no pronounced regulation was found on the time point 16 days.

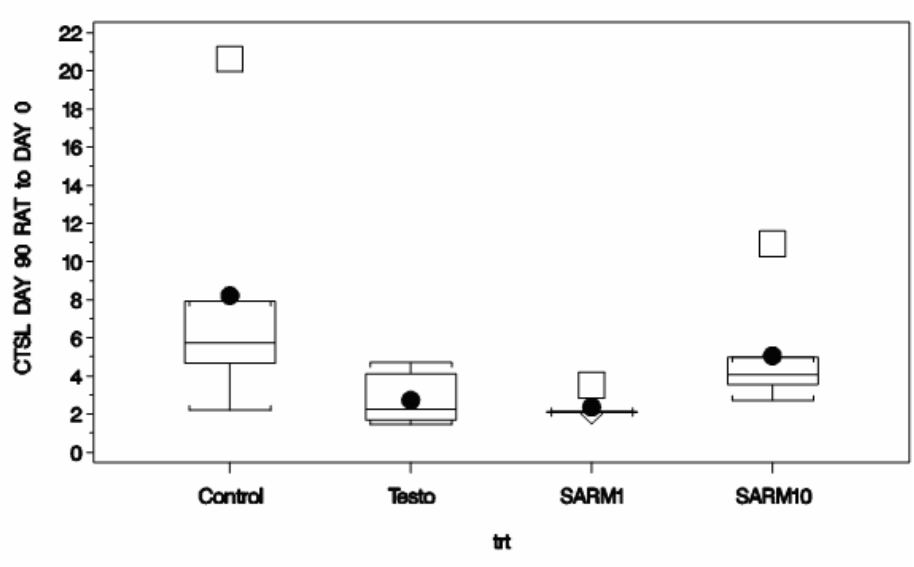


Figure 6: Significant down-regulation of CTSL between control and treated samples. Box-whisker plot show the expression ratio between the normalised expression of the timepoint 90 and baseline. The horizontal line inside the box indicates median and the box indicates the interquertile range. The full point indicates mean. The whiskers indicate the maximum and minimum and the squer indicates outliers. The calculation of the outliers is based on the box-whisker rule saying that a outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

The control group showed also high variability as compared to the treatment groups as indicated by the box-whisker plot. This reflects the natural variability of the non-induced expression in each studied subject. In contrast to the control group, in the treated groups also smaller variability was observed, possibly supporting the evidence for induced suppressing effect on the individual expression.

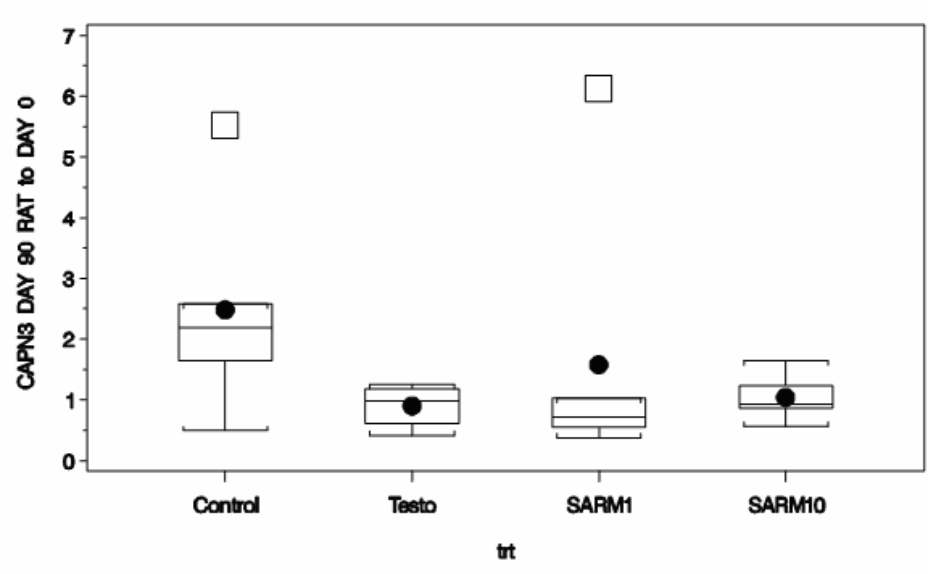


Figure 7: Significant down-regulation for CAPN3 between control and treated samples. Box-whisker plot show the expression ratio between the normalised expression of the timepoint 90 and baseline. The horizontal line inside the box indicates median and the box indicates the interquertile range. The full point indicates mean. The whiskers indicate the maximum and minimum and the squer indicates outliers. The calculation of the outliers is based on the box-whisker rule saying that a outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

In *m. quadriceps* the control samples already showed an expression with a high statistical spread in the significantly regulated candidate genes what mirrors natural differences between gene expressions in the individuals. This spread reduced in the treatment groups, showing a suppression of the individual expression differences induced by the treatment.

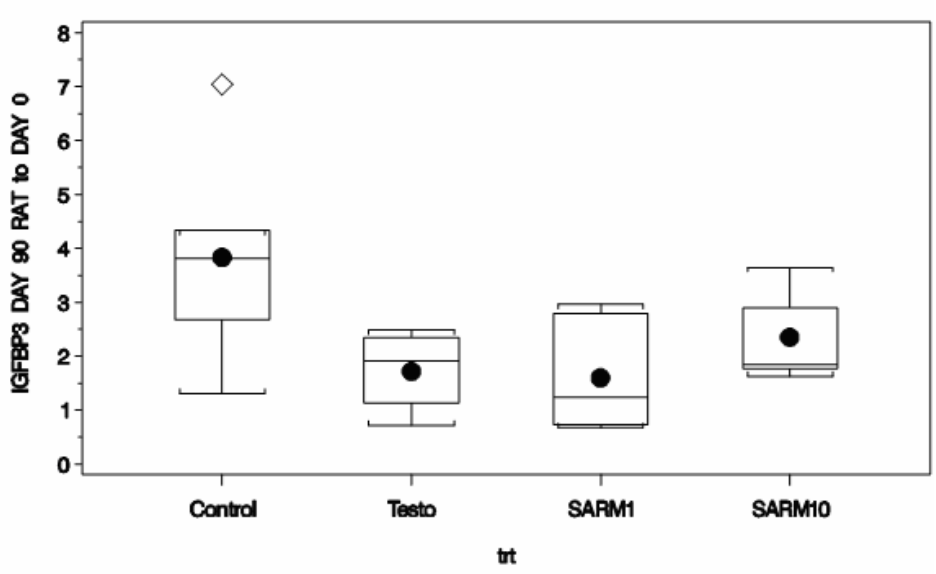


Figure 8: Significant down-regulation for IGFBP3 between control and treated samples. Box-whisker plot show the expression ratio between the normalised expression of the timepoint 90 and baseline. The horizontal line inside the box indicates median and the box indicates the interquertile range. The full point indicates mean. The whiskers indicate the maximum and minimum and the squer indicates outliers. *The diamond indicates weak outliers.* The calculation of the outliers is based on the box-whisker rule saying that a outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

Both the down-regulation of CTSL and IGFBP3 can be interpreted as first anabolic effects of the treatments in the muscle tissue. In the protein metabolism of the muscle CTSL expression characterises muscle wasting and IGFBP3 can inhibit the interaction of IGF1 and IGF1R which are important factors of muscle growth [84, 85, 86, 87]. With the down-regulation of both genes a positive effect on the protein anabolism and growth in the muscle can be supposed.

Down-regulations of CAPN3 could only be seen in conditions related to disuse and denervation of the muscle, an age-related phenomenon [88, 89, 90]. The animals were held in cages what could mirror the disuse of the muscle and explain the decrease of CAPN3 in the muscle tissue.

First influences of the treatments on the protein metabolism and the muscle growth could be seen via the significant expression of CTSL, CAPN3 and IGFBP3. These genes can be taken as the first biomarkers for a gene expression pattern of anabolic steroids.

3.3 Studies on human tissues

3.3.1 Cell culture experiments

RNA concentration and RNA integrity

Samples of the cell culture of the male donor showed higher mean±S.D. total RNA concentrations (86.2±41.2 ng/µl) than the samples of the cell culture of the female donor (51.4±8.3 ng/µl). In both cell cultures Stanozolol samples showed the highest concentrations (76.8±22.5 ng/µl; 144.7±35.1 ng/µl).

Samples taken after 6h treatment were analysed as mean reference for the RIN value. Mean RIN values were higher in the cell culture of the male donor (8.6, stanozolol; 9.9, testosterone; 7.5, nortestosterone; 8.4, clenbuterol) than in cell culture of the female donor (8.8, stanozolol; 8.1, testosterone; 5.0, nortestosterone; 5.1, clenbuterol).

Primer design

Not all of the tested primer pairs showed satisfactory results in melting curve analysis and in agarose gel electrophoresis. FasL, ERα, ERβ, Caspase 3 primer pairs were successfully established in human muscle and therefore also tested in human HFDPC. For these genes no specific product could be measured in HFDPC and also new designed primer pairs did not show a specific product. It can be supposed that these genes were not expressed in HFDPC. Therefore the results from these genes were not taken for statistical analysis. All other genes were taken for further calculations.

GROUP	IDENTITY		SEQUENCE 5´→ 3´	BP	AT (°C)	ACC.NR.
apoptosis	CAPN8	FOR	TGG CAC TGA TGG ACA GGA G	230	60	NM001228
		REV	GCA GAA AGT CAG CCT CAT CC			
	FasR	FOR	TTC TGC CAT AAG CCC TGT CC	174	60	NM000043
		REV	CCA CTT CTA AGC CAT GTC CTT C			
	Bcl-xl	FOR	TAA ACT GGG GTC GCA TTG TG	145	60	NM138578
		REV	TGG ATC CAA GGC TCT AGG TG			
	Bcl-2	FOR	GAG GAT TGT GGC CTT CTT TGA G	170	60	NM000633
		REV	ACA GTT CCA CAA AGG CAT CCC			
growth	GHR	FOR	ATC CAC CCA TTG CCC TCA AC	246	60	NM00163
factors		REV	ATC TCA CAC GCA CTT CAT ATT CC			
	IGF1R	FOR	CAT TTC ACC TCC ACC ACC AC	151	60	NM000875
		REV	AGG CAT CCT GCC CAT CAT AC			
	IGFBP3	FOR	CAC AGA TAC CCA GAA CTT CTC C	227	60	M35878

		REV	CCA TAC TTA TCC ACA CAC CAG C			
	IGFBP5	FOR	GAG CTG AAG GCT GAA GCA GT	177	55	ENST0233813
		REV	GAA TCC TTT GCG GTC ACA ATT			
cell cycle	Cyclin D1	FOR	AAC AAG CTC AAG TGG AAC CTG G	170	60	NM053056
		REV	CAT GGA GGG CGG ATT GGA AAT			
	Cyclin E	FOR	CTA TCC TCC AAA GTT GCA CCA G	259	60	AF518727
		REV	CAA GGC AGT CAA CAT CAA GGA			
enzymes	HSD17b1	FOR	TGG ACG TCT TGG AGC TTT GTG	233	58	ENS0225929
		REV	CAG ATC TGT CTG GGT CAG CA			
	SRD5A1	FOR	CTT GAG CCA TTG TGC AGT GT	166	58	ENST0233239
		REV	GCC TCC CCT TGG TAT TTT GT			
	SRD5A2	FOR	TGA ATA CCC TGA TGG GTG GT	181	60	ENST0233139
		REV	GGA AAT TGG CTC CAG AAA CAT A			
hair	FGF2	FOR	AGA AGA GCG ACC CTC ACA TC	237	60	M27968
metabolism		REV	ACT GCC CAG TTC GTT TCA GT			
	FGF7	FOR	CCT GAG CGA CAC ACA AGA AG	167	60	M60828
		REV	GCC ACT GTC GCT TCC TTA TT			
	FGF1R	FOR	GAA GTT CAA ATG CCC TTC CAG TG	215	58	ENST0310729
		REV	CCA GCT GGT ATG TGT GGT TG			
	FGF2R	FOR	CAG AGA CCA ACG TTC AAG CA	196	58	ENST0336553
		REV	GAG GAA GGC ATG GTT CGT AA			
	HGF	FOR	CTG GTT CCC CTT CAA TAG CA	171	58	ENST0222390
		REV	CTC CAG GGC TGA CAT TTG AT			
	NT5	FOR	AGG CTG ATA ACG CTG AGG AAG	105	60	NM006179
		REV	CAT AGG ACT GCT TGG CCT TG			
steroid	AR	FOR	TTG TCC ATC TTG TCG TCT TCG G	237	60	L29496
receptors		REV	TGT CCA GCA CAC ACT ACA CC			
	GR	FOR	TTC TGC GTC TTC ACC CTC AC	159	60	AH002750
		REV	CTG TCT CTC CCA TAT ACA GTC C			
reference	UBC	FOR	TGA AGA CTC TGA CTG GTA AGA CC	128	60	NM021009
genes		REV	CAT CCA GCA AAG ATC AGC CTC			
	GAPDH	FOR	GAA GGT GAA GGT CGG AGT CAA	233	60	NM002046
		REV	GCT CCT GGA AGA TGG TGA TG			
	ACTB	FOR	AGTCCTGTGGCATCCACGAAAC	78	60	NM01101
		REV	GCAGTGATCTCCTTCTGCATCC			

Tabel 11: Primer sequences of genes analysed in HFDPC and hair follicle samples. Amplicon lengths (BP) and annealing temperatures (AT) are listed. Not all genes could be measured in the experiments because of the limited RNA amount.

qRT-PCR and gene expression regulations in HFDPC

The results of the qRT-PCR runs showed high variations between the replicates (>1.5 cycles) for the testosterone, nortestosterone and clenbuterol treatment. It can be supposed that the rather low total RNA amount of several samples was the reason for the high variations in the Ct values. To avoid speculations and false-positive results only the constant Ct values of the stanozolol treatment were taken for further statistical analysis.

Gen expression analysis within one treatment time point (ΔΔCt)

In both cell cultures AR showed a significant down regulation at 24h and FasR an up-regulation at 6h. In the cell culture of the female donor the 71% down regulation of AR was seen in the 1nM concentration (p-value=0.047), in the cell culture of the male donor with 35% in the 100nM concentration (p-value=0.014). FasR was up-regulated at 6h for 10nM concentration in the cell culture the female donor (55%, p-value=0.023) and in the cell culture of the male donor (720%, p-value=0.049). Additionally FGF7 was down-regulated at 0h in the 100nM concentration (85%, p-value=0.009) and 10nM concentration (65%, p-value=0.046) and at 6h in the 100nM concentration (94%, p-value=0.03) in the cell culture of the female donor. In the cell culture of the male donor SRD5A2 (6h, p-value=0.028) and FGF2 (0h, p-value=0.010) showed a significant down-regulation of 83% and 15%in the 100nM concentration.

Gen expression over treatment time (2-Way-Anova)

With the 2-Way-Anova one significant correlation of Caspase 8 to treatment concentration and treatment time could be calculated in the cell culture of the female donor. Caspase 8 in the 1nM concentration was significantly different from all other concentrations and significant differences between 0h to 6h and 0h to 48h could be measured for this gene.

	Stanozolol	
	time	**treatment**
AR	0.751	0.312
FasR	0.137	0.057
FGF2	0.203	0.471
FGF7	0.374	0.959
IGF1R	0.095	0.181
Caspase8	**0.028**	**0.010**

Tabel 12: P-Values of 2-Way-Anova calculations of treatment and treatment time points. Caspase 8 showes significant differences between the treatment groups and the treatment time points (p-value< 0.05).

In both cell cultures AR and FasR showed the similar regulation under stanozolol treatment at the same treatment time point. FasR additionally showed same regulations at the same concentration. Therefore, AR and FasR could be taken as first biomarkers for a gene expression pattern of stanozolol treatment.

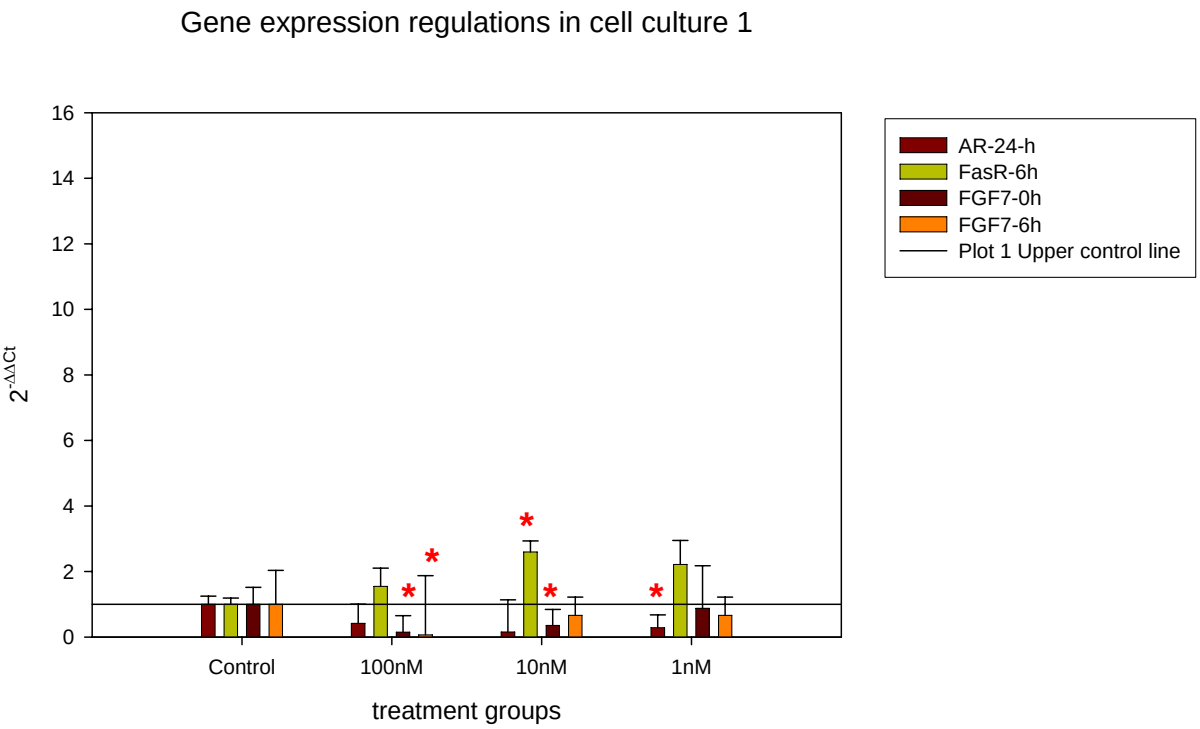


Figure 9: Significant gene expression regulations in the cell culture of the female donor. The ratio was calculated by $2^{-\Delta\Delta Ct}$ whereby the control was set 1 (upper control line). Data are depicted in bars ± standard deviations. Red asterisks (*) mark the significantly regulated target genes.

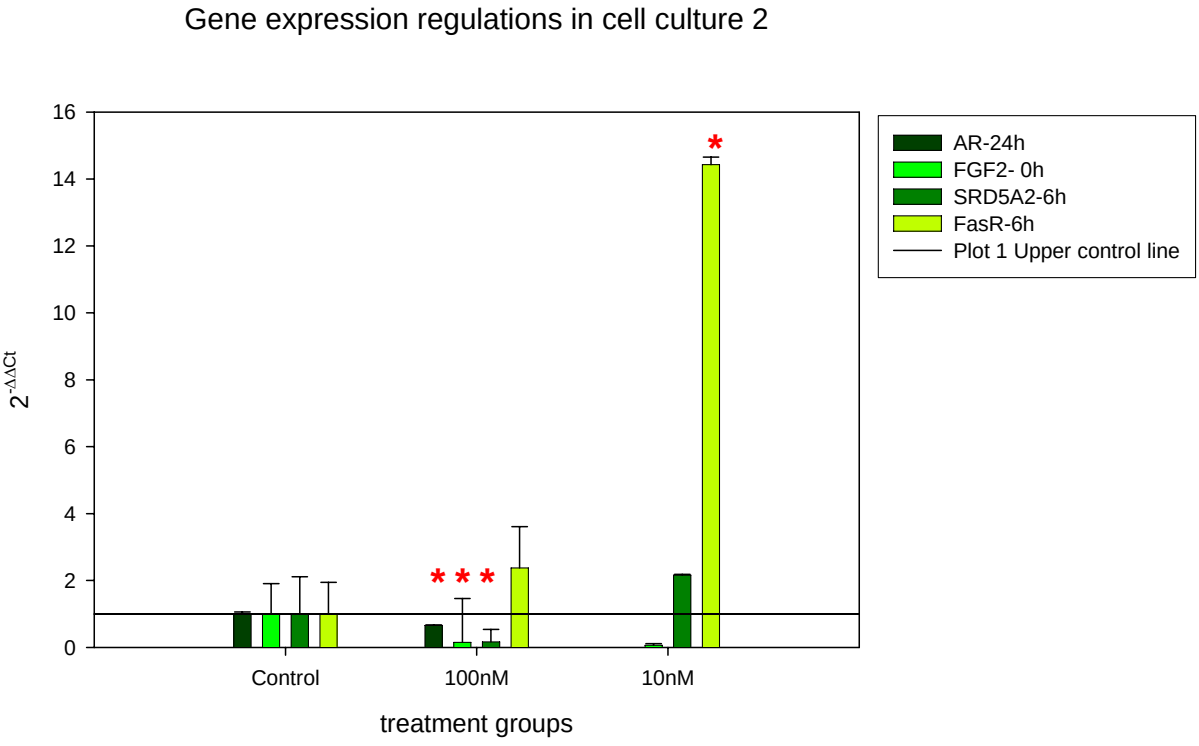


Figure 10: Significant gene expression regulations in the cell culture of the male donor. The ratio was calculated by $2^{-\Delta\Delta Ct}$ whereby the control was set 1 (upper control line). Data are depicted in bars ± standard deviations. Red asterisks (*) mark the significantly regulated target genes.

A first hint that also the growth phase of the hair follicle influences gene expressions could be seen in the different regulations of FGF2 and FGF7 in the untreated samples in both cultures. FGF7 is known to induce the growth phase (anagen phase) in the hair follicle, whereas FGF2 could be taken as its antagonist by inhibiting the morphogenesis [48]. In the cell culture of the female donor FGF7 was significantly down-regulated what would show that these cells were taken from a hair follicle in the catagen phase. In the cell culture of the male donor FGF2 was significantly down-regulated, indicating the anagen phase. Several studies identified this factor in the hair follicle but this was the first time that also an expression in the hair papilla could be measured [45, 46, 47].
The significant regulations of FasR and Caspase 8 in the cell culture of the female donor showed first influences on metabolic pathways. The regulation of both factors could indicate a possible influence of stanozolol on the extrinsic way of apoptosis.

In this study AR and FasR were identified as first biomarkers for a gene expression pattern for stanozolol treatment. Caspase 8 and FGF2 and FGF7 were not significantly regulated but expressed in the hair papilla what makes the steroid receptor, apoptosis and hair cycle group to interesting functional groups for further analysis. Physiological influences can be supposed in the apoptosis and hair growth regulation. The results of this study were taken for further analysis in hair follicle samples.

3.3.2 Hair follicle experiments

Histological stains

Histological stains of plucked hair follicle samples clearly showed that cells of the root sheath, especially the outer root sheath could be gained and used for RNA extraction without taking a skin biopsy. It was not possible to gain the hair papilla by plucking as expected because it remains to build up a new hair shaft.

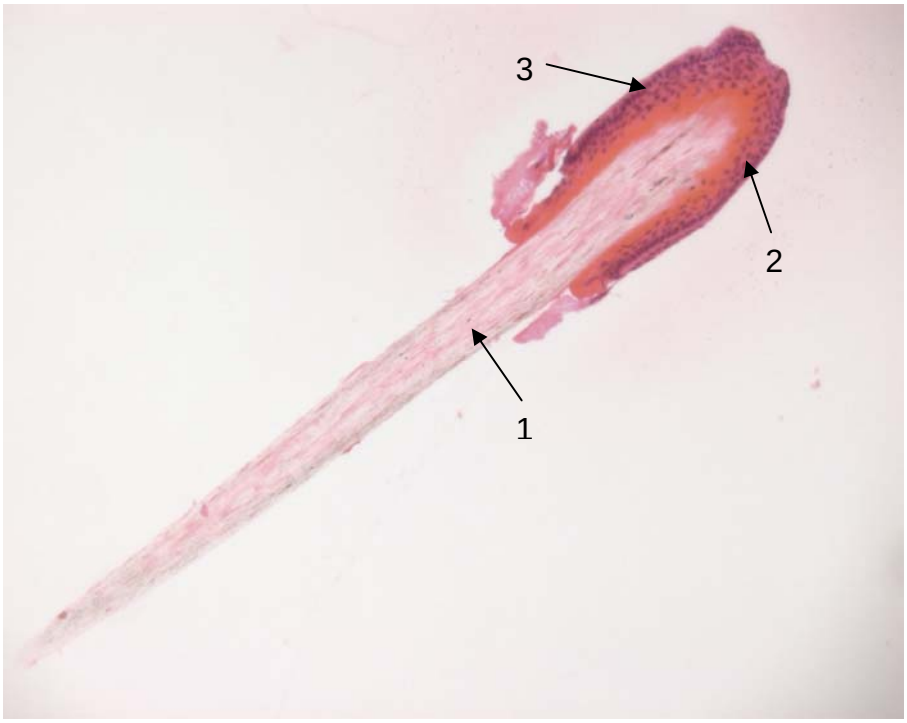


Figure 11: Slice of the plucked hair with the hair shaft (1) and outer root sheath cells (2) which are clearly outlined by perifollicular sheath cells (3), stained in blue.

RNA concentration and RNA integrity

Mean total RNA concentration of all samples of the first study was 67.5±52.0 ng/µl, in the second study 102.5±80.1 ng/µl.

In the first study six male and six females samples were taken for mean RIN analysis that was 4.4. In study two the control and treated samples showed a mean RIN of 7.0.

The total RNA extraction of the hair follicle samples of the second study was done directly after the plucking, in the first study samples were first frozen in liquid nitrogen, stored at

-80°C for some days until extraction was done. The freezing seems to damage cells so that RNA is partly destroyed or degraded, what results in a lower RNA yield.
This explains the differences in the RNA concentrations and RIN values between the samples of the first and the second study.

Primer design

The primer pairs designed for all measured target genes were first established in human hair follicle dermal papilla cell samples or primate muscle tissue. Like in the HFDPC, ERα and Caspase 3 did not show specific results in melting curve analysis. AR, FGF2, SRD5A2 and Caspase 8 also showed no specific products even though the genes were measured in HFDPC. It is supposed that these genes were not expressed in the hair follicle cells, what is possible because it is known that androgens act via the hair papilla and induce gene expressions in the hair follicle afterwards.
In the first study the reference genes GAPDH and UBC and in the second study the reference genes ACTB and UBC had constant expression levels and could be taken for statistical calculation.

Results and discussion of qRT-PCR and gene expression regulations in hair follicles

Following target genes were measured in the hair follicle experiments:

TG	Study 1	Study 2
AR	×	×
ERα	×	×
GR	✓	✓
FGF7	n.m.	✓/×
FGF2	×	n.m.
Caspase 8	×	×
FasR	✓	✓
Bcl-2	✓	n.m.
SRD5A2	×	n.m.
IL1β	✓	n.m.

Tabel 13: Target genes measured in both hair follicle experiments. Study 1 included male and female samples; in study 2 samples of treated and untreated men were measured. n.m. means not measured target genes, ✓ showed specific products and × showed unspecific products in the PCR run. All TG with a × were not taken for statistical analysis.

FGF7 only showed a specific product in the treated samples of study two, in the control samples the melt curve analysis showed no specific peak.

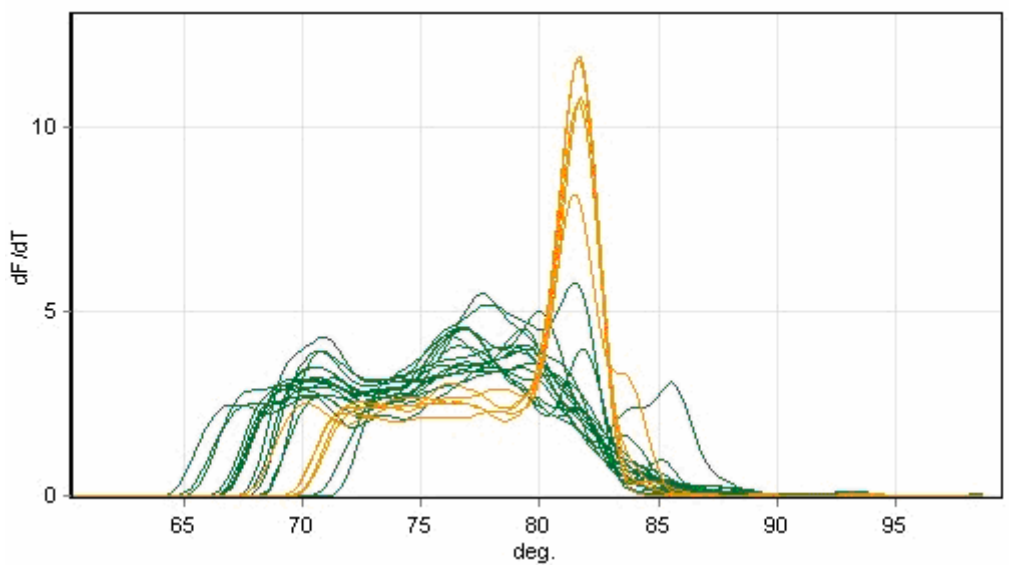


Figure 12: For FGF7 a specific PCR product could only be seen in the treated samples (orange), not in control samples (green). On the x-axis the temperature in °C and on the y-axis the first derivative of the melt curve is shown.

In study 1 no significant differences between gene expressions in the female and male samples could be identified.
In the second study a first androgen dependent effect on the gene expression could be seen for FGF7. Looking at the specific melt peak of treated and untreated hair follicle samples (Figure 12), FGF7 was only expressed in the treated samples (mean Ct = 23.9 cycles). Additionally GR was significantly down-regulated (41%; p-value=0.02) in the treatment samples. It is known that androgens can bind and act via the GR what induced the significant regulation in this study. The expression of IL1β and Bcl-2 in the first study and FasR in both studies in the inner and outer root sheath of the hair follicle could be approved. [46, 91, 92].

These results show first promising differences between treated and untreated samples, a precondition for the development of a possible screening method for anabolic agents. FGF7 GR, IL1β, Bcl-2 and FasR were identified as first potential biomarkers for anabolic agents in hair follicle samples. Different gene expressions between HFDPC and hair follicle samples were expected because it is known that androgens act via the hair papilla and influence factors in the hair follicle afterwards [93, 94, 95, 96, 97]. Therefore the hair papilla would be the more promising tissue for gene expression analysis. Because it can not be taken by plucking, only by skin biopsy, it is not suitable as potential doping control tissue. This would only be possible with the hair follicle samples but further studies are necessary to show if the influences of AAS in this tissue are still strong enough to induce constant gene expressions.

4. Conclusion

All the various studies that are included in this thesis clearly show that qRT-PCR seems to be a highly specific and sensitive method to analyze gene expressions in various tissues and species. The most important part of a study and therefore precondition of a successful data analysis is sampling, RNA extraction and purification in the beginning of the experiment. Especially in samples with low RNA yield, a high RNA quality is important for good results.
In all experiments the relative quantification of gene expression was done, using reference genes for normalization. This method is accepted for gene expression analysis but bears the risk that the chosen reference genes are also significantly regulated. Therefore it is extremely important to proof the non-regulation of the reference genes to avoid false interpretations.
To search for relevant target genes in the beginning of the study using literature shaped up as a very efficient approach in tissues like the uterus and muscle. Here the influence of AAS was often investigated and most of the selected target genes could be amplified and showed significant regulations. In some tissues, e.g. the hair follicle the influence of anabolic agents might not be established as well as in the uterus or the muscle. For such tissues a pre-analysis of target genes using a hybridisation microarray could be helpful.
The experiments also showed that it is more promising to choose tissues that are directly influenced by anabolic hormones, such as the uterus. Here the regulations of the biomarkers showed very high expressions differences. It is recommended that further animal studies to identify biomarkers for treatment with anabolic agents should be done in primary sexual organs, e.g. the uterus, testis, prostate, sperm, or in blood. This is also applied for the human system but here only very few tissues can be taken for such analysis, e.g. blood or hair. The blood seems to be an auspicious tissue because it can easily be taken in every species and higher gene expression regulations can be expected under the influence of anabolic agents. Taking the hair for such analysis, the hair papilla would be more promising than the hair follicle.
The aim to identify biomarkers, showing same regulations under a specific treatment in various tissues could partly be obtained because biomarkers have to be optimized for each tissue. Also factors like the health status, nutrition or environmental conditions seem to influence gene expression regulations. This should be kept in mind when gene expression regulations are interpreted. The number of animals or samples taken for further studies has to be much higher to make organism specific differences as low as possible.

Summarizing all biomarkers for a treatment pattern of anabolic agents that were found in the different tissues, the steroid receptors, protein metabolism factors and apoptosis factors seem to be universal biomarkers, expressed in every tissue and species. Also tissue specific biomarkers, such as the adipolysis factors in the liver, the muscle proteins in the muscles and the glycolysis factors in the uterus, could be identified.

It also seems that the treatment with AAS results in a general suppression of the natural, individual gene expressions.

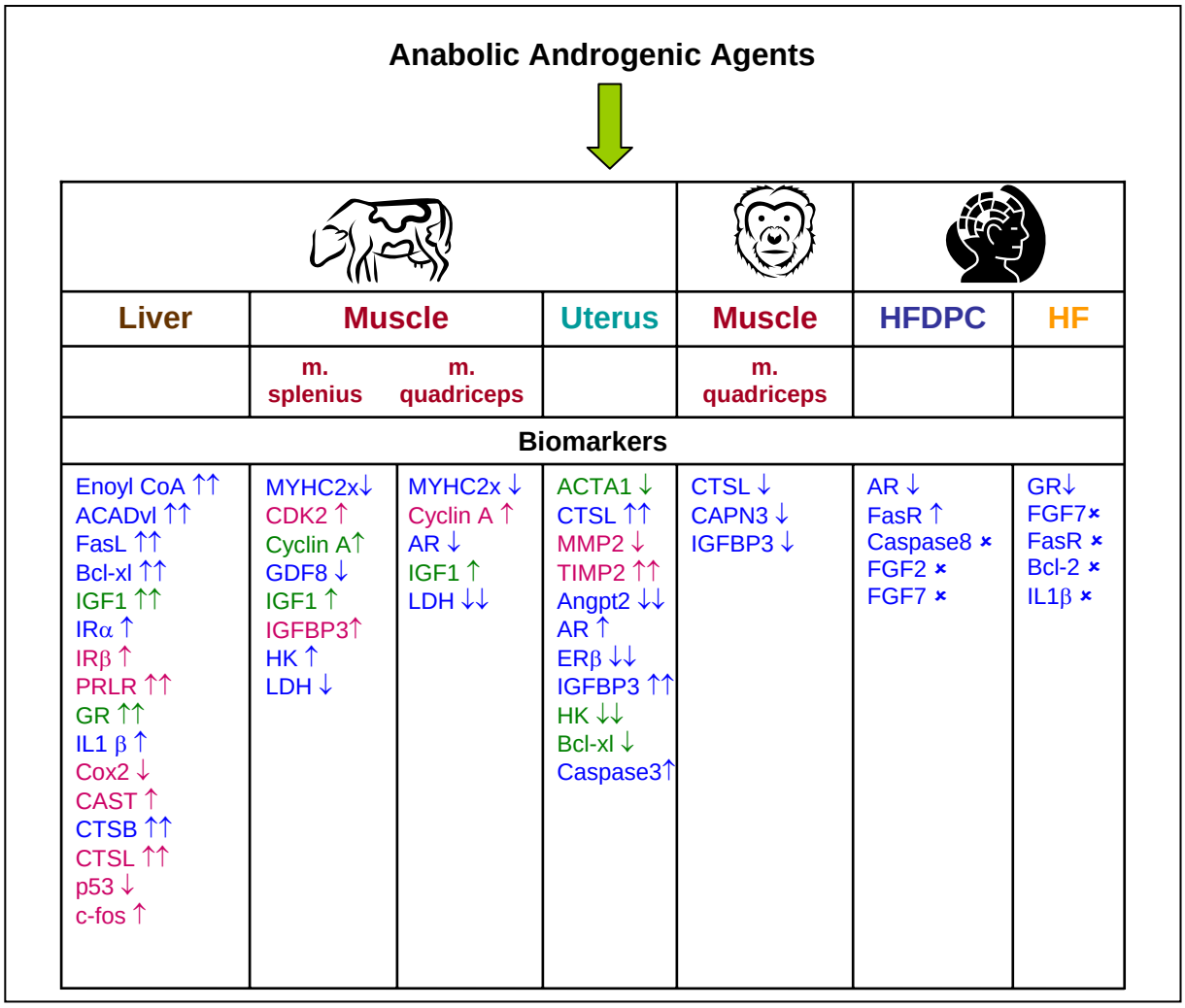


Figure 13: List of identified biomakers for a treatment pattern of anabolic agents in the different tissues. ↓ shows a down-regulation, ↑ an up-regulations, one arrow shows p-value<0.05, two arrows show p-value<0.01. × means that no significant expression could be calculated (no control group) but these target genes were also expressed in the tissue and could be taken as possible biomarkers. TG colored in blue were significantly regulated under androgen treatment, in green under estrogen treatment and in purple under gestagen treatment.

Physiological effects of AAS are supposed to influence the extrinsic apoptosis pathway. In this signal cascade several factors were significantly up-regulated and anti-intrinsic factors down-regulated (Figure 14).

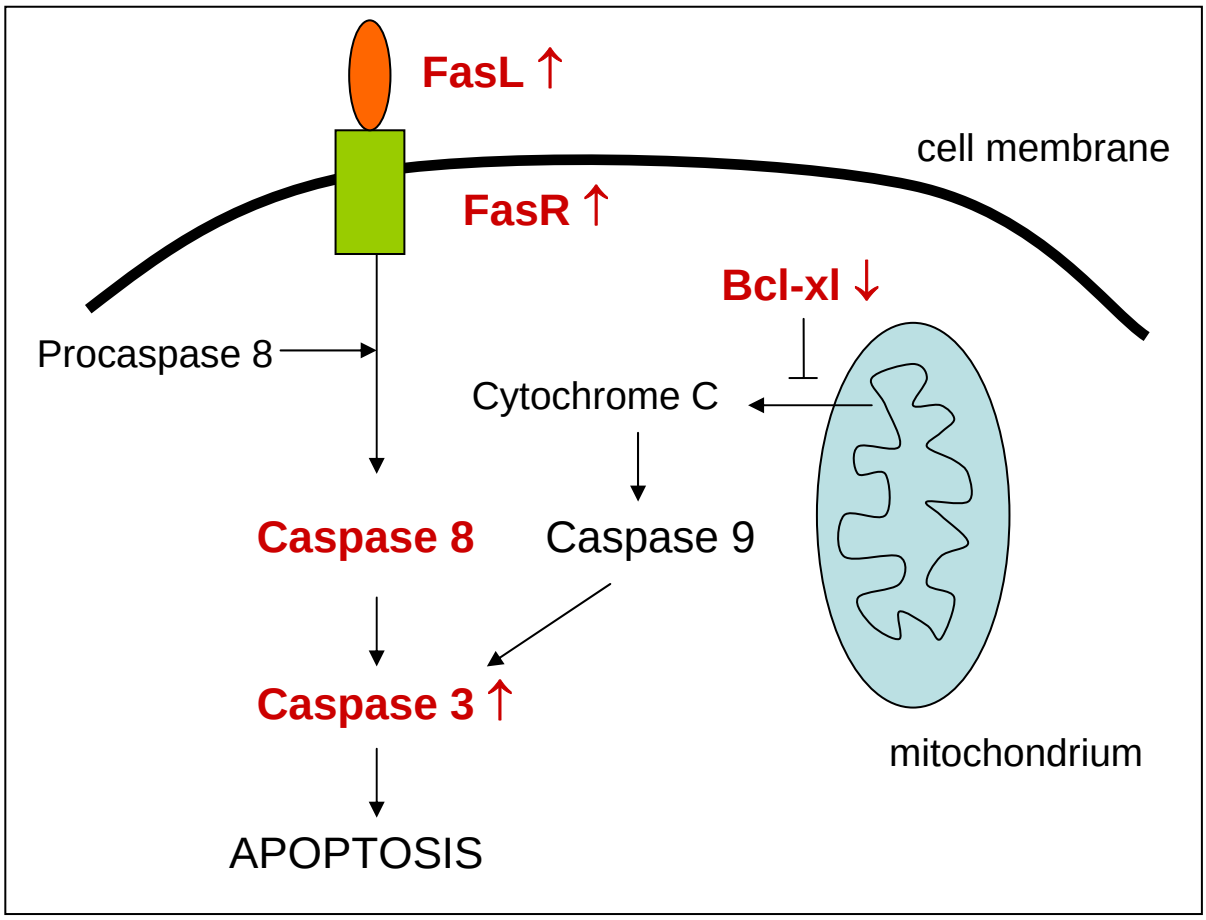


Figure 14: Signalling pathway of apoptosis. Genes coloured in red are part of the extrinsic way and were significantly regulated in all treated tissues.

This thesis showed that the analysis of AAS via gene expression biomarkers is possible in specific tissues and could be used to identify physiological changes in the organism. First steps toward a screening pattern for anabolic agents could be done. The development of a universal, bovine screening pattern is not recommended because the biomarkers have to be optimized for each specific tissue. It should be possible to use the identified tissue specific biomarkers in different species. Modifications of the apoptosis pathway, the glycolysis, the β-oxidation and protein metabolism seem to show first metabolic effects of AAS in the organism.

It has to be pointed out that all studies done for this thesis were feasibility studies and first steps toward a possible new screening method. Further studies are necessary to find more biomarkers for a screening pattern of anabolic agents and to identify complete metabolic pathways.

The ambitions for the future should be the identification of specific gene patterns for functional groups of anabolic agents in one tissue, in order that abuses can be uncovered.

Reference List

1 The prohibited list 2007. International standard. World anti-doping agency. 2006.

2 Clasing D, Müller RK. Dopingkontrolle – Informationen für Aktive, Betreuer und Ärzte zur Bekämpfung des Medikamentenmissbrauchs im Sport. Bundesinstitut für Sportwissenschaften. 4., überarbeitete Auflage, 2006.

3 Saudan C, Baume N, Robinson N, Avois L, Mangina P, Saugy M. Testosterone and doping control. *Br J Sports Med*, 2006, 21-24.

4 Lange I, Meyer HD. Hormone. In: Handbuch der Lebensmitteltoxikologie, Band3 von 5, (Dunkelberg H, Gebel T, Hartwig A, eds,):Wiley-VCH Verlag Weinheim, ISBN 978-3-527-31166-8, S. 1579-1608, 2007.

5 Homepage des Instituts für Biochemie der DSHS Köln. 15.01.2005
www.dopinginfo.de

6 Ayotte C. Significance of 19-norandrosterone in athletes´urine samples. *Br J Sports Med*, 2006, 40:25-29.

7 Meyer HD. Estrogens and Antiestrogens. In: Handbook of Experimental Pharmacology (Oettel, M. and Schillinger, E., eds.), Volume 135/II. Springer-Verlag, Berlin, Heidelberg, 1999.

8 Pottier J, Cousty C, Heitzman RJ, Reynolds IP. Differences in the biotransformation of a 17 beta-hydroxylated steroid, trenbolone acetate, in rat and cow. *Xenobiotica*, 1981, 11, 489-500.

9 Randall D, Burggen W, French K, Eckert WH. Animal Physiology, Freeman and Company 2002.

10 Kleinova M, Zoellner P, Kahlbacher H, Hochsteiner W, Lindner W. Metabolic profiles of the mycotoxin zearalenone and of the growth promoter zeranol in urine, liver, and muscle of heifers. *J Agric Food Chem*, 2002, 50, 4769-4776.

11 Meyer HHD. Bewertung von Wirkstoffen aus der Tierhaltung als mögliche Dopingsubstanzen im Humansport – In: Entwicklung in der Dopingforschung. Wissenschaftliche Berichte und Materialien; Bundesinstitut für Sportwissenschaften, (Müller-Platz C. eds.), Sportverlag Strauß, Köln, Band 08, ISBN 3-939390-87-9, S. 77-85, 2006.

12 Meyer HHD, Biochemistry and physiology of anabolic hormones used for improvement of meat production. *APMIS*, 2001 109, 1- 8

13 Meyer HHD, Lange IG. β-Agonisten. In:Handbuch der Lebensmitteltoxikologie, Band3 von 5, (Dunkelberg H, Gebel T, Hartwig A, eds,):Wiley-VCH Verlag Weinheim, ISBN 978-3-527-31166-8, S. 1579-1608, 2007.

14 Baron DA, Martin DM, Magd SA. Doping in sports and is spread to at-risk populations: an international review. *World Psychiatry*, 2007, 6:2.

15 Kuhn CM. Anabolic Steroids. *Recent Prog Horm Res*, 2002, 57:411-34.

16 Brook R. Doping in Sport: Misuse, analytical tests, and legal aspects. *Clin Chem*, 1997, 43:7.

17 Pallesen S, Jøsendal O, Johnsen BH, Larsen S, Molde H. Anabolic steroid use in high school students. *Subst Use Misuse*, 2006, 41(13):1705-17.

18 VandenBerg P, Neumark-Sztainer D, Carfi G, Wall M. Steroid use among adolescents: longitudinal findings from project EAT. *Pediatrics*, 2007, 119(3):476-86.

19 Apple JK, Dikemann ME, Simms DD, Kuhl G. Effects of synthetic hormone implants, singularly or in combination, on performance, carcass traits and longissimus muscle palatability of Holstein steers. *J Anim Sci*, 1991, 69:4437-4448.

20 Hartel A. Biologisches Screening anaboler Wirkstoffe – In: Entwicklung in der Dopingforschung, Wissenschaftliche Berichte und Materialien; Bundesinstitut für Sportwissenschaften, (Müller-Platz C eds.) Sportverlag Strauß, Köln, Band 08, (2006), ISBN 3-939390-87-9, S. 65-76.

21 Food and Agriculture Organization of the United Nations. Joint FAO/WHO expert committee on food additives (JECFA). FAO Roster of experts for JECFA for residue of veterinary drugs in food (2007-2001). 28.09.2007
http://jecfa.ilsi.org/

22 European Commission, Directorate General Health and Consumer Protection. Guidance document on residue analytical methods. SANCO/825/00 rev. 17/03/2004.

23 Bundesamt für Verbraucherschutz und Lebensmittelsicherheit. Tierarzneimittelrückstände in Lebensmitteln.05.09.2005.
http://www.bvl.bund.de/cln_027/nn_493968/DE/05__Tierarzneimittel/02__RueckstInLm/rueckst__LM__node.html__nnn=true

24 Handelsman DJ. Testosterone:use, misuse and abuse. *MJA*, 2006, 185(8):436-439.

25 Yin D, Gao W, Kearby JD, Xu H, Chung K, He Y, Marhefka CA, Veverka KA, Miller DD, Dalton JT. Pharmacondynamics of selective androgen receptor modulators. *J Pharmacol Eperim Therap*, 2003, 304(3):1334-1340.

26 Bross R, Storer T, Bashin S. Aging and Muscle loss. *TEM* 1999, 10(5): 194-198.

27 Muller M, Grobbee DE, Thijssen JHH. Sex hormones and male health: effects on components of the frailty syndrome. *Trends Endocrinol Metabol,* 2003, 14(6): 289-296.

28 Van den Beld A, Huhtaniemi IT, Pettersson KS et al. Luteinizing Hormone and Different Genetic Variants, as Indicators of Frailty in Healthy Elderly Men, *JCEM,* 1998, 84(4): 1334-1339.

29 Yialamas MA, Hayer FJ. Androgens and the ageing male and female. Best Practice and Research, *Clin Endocrin and Metabol,* 2003, 17(2): 223-226.

30 Davis S. Androgen Replacement in Women: A Commentary. *JCEM,* 1999, 84(6): 1886-1891.

31 Davis SR. The therapeutic use of androgens in women. *J St Biochem Mol Biol,* 1999, 69:177-184.

32 Deschenes MR. Effects of aging on muscle fibre type size. *Sports Med,* 2004, 34(12): 809-24.

33 Doherty TJ. Invited review: aging and sarcopenia. *J Appl Physiol*, 2003, 95(4): 1717-27.

34 Lopez FJ. New approaches to the treatment of osteoporosis. *Curr Opin Chem Biol,* 2000, 4 (4):383-393.

35 Chen J, Hwang DJ, Bohl CE et al. A selective androgen receptor modulator (SARM) for hormonal male contraception. *JPET,* 2005, 312: 546–553.

36 Negro-Vilar A. Selective Androgen Receptor Modulators (SARMs): A Novel Approach to Androgen Therapy for the New Millennium. *JCEM,* 1999, 84(10): 3459-3462.

37 Yin D, Gao W, Kearbey JD. Pharmakodynamics of Selective Androgen Receptor Modulators. *JPET* 2003, 304(3): 1334-1340.

38 Rosen J, Negro-Vilar A. Novel, non-steroidal, selective androgen receptor modulators (SARMs) with anabolic activity in bone and muscle and improved safety profile. *J Musculoskelet Neuronal Interac,*. 2002, 2(3):222-4.

39 Catlin DH, Hatton CK, Starcevic SH. Issues in detectin abuse of xenobiotic anabolic steroids and testosterone by analysis of athletes´urine. *Clinical Chemistry*, 1997, 43(7):1280-1288.

40 U.S.A Food and Drug Administration. Surrogate Markers – Their role in regulatory decision progress. 21.08.2003
http://www.fda.gov/cder/Offices/Biostatistics/Chakravarty_376/sld016.htm

41 Rehner G, Daniel H. Biochemie der Ernährung. 2. Auflage, Spektrum Akad. Verlag, 2002.

42 Dorak MT. Real-time PCR.. 1st ed., Taylor and Francis Group, 2006.

43 Darbre P, Yated J, Curtis S, King RJB. Effect of estradiol on human breast cancer cells in culture. *Cancer Res*, 1983, 43:349-354.

44 Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. *Mol A Med* 2006, 27: 126-139.

45 Schroeder A, Mueller O, Stocker S et al.The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol,* 2006, 31(7): 3.

46 Kirschke H, Barrett AJ, Rawlings ND. Proteinases 1: lysosomal cysteine proteinases. 2nd ed. New York: Oxford University Press; 1998, 13–18.

47 Kugler P, Vornberger G. Renal cathepsin-B activities in rats after castration and treatment with sex hormones. *Histochemistry*, 1986, 85(2), 157-61

48 Reeds PJ, Hay SM, Dorward PM, Palmer RM. Stimulation of muscle growth by clenbuterol: lack of effect on muscle protein biosynthesis. *Br. J. Nutr*. 1986,56, 249.

49 Voet D, Voet JG, Pratt CW, in: Lehrbuch der Biochemie (Wiley-VCH). 2002.

50 Penzlin H, Lehrbuch der Tierphysiologie; 7. Auflage. Elsevier, Spektrum Akademischer Verlag, 2005.

51 Vandekerckhove J, Bugaisky G, Buckingham M. Simultaneous expression of skeletal muscle and heart actin proteins in various striated muscle tissues and cells. A quantitative determination of the two actin isoforms. *J Biol Chem*, Feb 5;261(4), 1986, 1838-1843.

52 Vandekerckhove J, Weber K. Chordate muscle actins differ distinctly from invertebrate muscle actins. The evolution of the different vertebrate muscle actins. *J Mol Bio,* Dec 25; 126(4), 1978, 783-802.

53 Hunt DW, Henricks DM, Skelly GC, Grimes LW. Use of trenbolone acetate and estradiol in intact and castrate male cattle: effects on growth, serum hormones, and carcass characteristics. *J Anm Sci* 69, 1991, 2452- 2462.

54 Ho JW, Song JZ, Leung YK . Activation of p53 by specific agents in potential cancer therapy. Curr *Med Anti-Canc Agents*, 2005, 5(2): 131-135.

55 Ishiguro N, Ohzono T, Shingawa T, Horiuchi M, Shingawa M. A spontaneous internal deletion of the c-myb protooncogene enhances transcriptional activation in bovine T lymphoma cells. *The Journal of Biological Chemistry*, 1994, 269(43): 26822-26829.

56 Kleinova M, Zoellner P, Kahlbacher H, Hochsteiner W, Lindner W. Metabolic profiles of the myotoxin zearalenone and of the growth promoter zeranol in urine, liver, and muscle of heifers. J *Agric Food Chem*, 2002, 50: 4769-4776.

57 Pfaffl MW, Lange IG, Daxenberger A, Meyer HHD. Tissue-specific expression of estrogen receptors (ER): Quantification of ERα and ERβ mRNA with real-time RT-PCR. *APMIS*, 2001, 109: 345-355.

58 To YC. The effect of neonatal administration of sex hormones on ribonucleic acid metabolism in the liver of male and female rats. *Br J Cancer*, 1971, 25: 516-519.

59 Ferrara N. Vascular endothelial growth factor and the regulation of angiogenesis. *Recent Prog Horm Res*, 2000; 55:15-35; discussion 35-36.

60 Lee S-J and McPherron AC. Myostatin and the control of skeletal muscle mass. *Curr Op Gene*., 1999, Dev. 9:604–607.

61 McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature*, 1997, 387: 83–90.

62 Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. *J Biol Chem,* 2002, 277:49831-49840.

63 Shinha-Hikim I, Cornford M, Gaytan H, Lee ML, Bashin S. Effects of testotserone supplementation on skeletal muscle fibre hypertrophy and satellite cell in community-dwelling older men. *J Clin Endocrinol Metab,* 2006, 91:3024-3033.

64 Vandekerckhove J, Bugaisky G, Buckingham M. Simultaneous expression of skeletal muscle and heart actin proteins in various striated muscle tissues and cells. A quantitative determination of the two actin isoforms. *J Biol Chem,* 1986, 261:1838-1843.

65 Bartoli M, Richard I. Calpains in muscle wasting. *Int. J. Biochem. Cell Biol.* 2005, 37:2115-2133.

66 Dargelos I, Moyen, C, Dedieu S, Veschambre P, Poussard S, Vuillier-Devillers K, Brustis JJ, Cottin P. Development of an inducible system to assess p94 (CAPN3) function in cultured muscle cells. *J. Biotechnol.* 2002, 96:271-279.

67 Degterev A, Boyce M, Yuan JY. A decade of caspases. *Oncogene* 2003, 22:8543-8567.

68 Baxter RC. Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *Am J Physiol Endocrinol Metab,* 2000, 278:E967-E976.

69 Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J Biol Chem* 1997, 272:6653-6662.

70 Florini JR, Ewton DZ, Coolican SA. Growth hormone and the insulin-like growth factor system in myogenesis. *Endocr Rev,* 1996, 17:481-517.

71 Beauchamp JR, Heslop L, Yu DS, Tajbakhsh S, Kelly RG, Wernig A, Buckingham ME, Partridge TA, Zammit PS. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J Cell Biol,* 2000, 151:1221-1234.

72 Mitusi S, Ohuchi A, Hotta M, Tsuboi R, Ogawa H. Genes for a range of growth factgors and cyclin-dependent kinase inhibitors are expressed by isolated human hair follicles. *Br J Dermato*l, 1997, 137: 693-698.

73 Ota Y, Sioth Y, Suzuki S, Ozawa K, Kawano M, Imamura T. Fibroblast growth factor 5 inhibits hair growth by blocking dermal papilla cell activation. *Biochem Biophys Res Commun*, 2002, 11;290(1):169-76.

74 Schlake T. FGF signals specifically regulate the structure of hair shaft medulla via IGF-binding protein 5. *Development*, 2005, 132: 2981-2990.

75 Stenn KS, Paus R. Controls of hair follicle cycling. *Pharm Rev*, 2001, 81 (1).

76 Ensemble Genome Browser. Ensembl release 48, 2007. http://www.ensembl.org

77 National Center for Biotechnology Information. Revised: January 25, 2008. http://www.ncbi.nlm.nih.gov

78 Primer 3 (v0.4.0). Feburary 07, 2007. http://www.frodo.wi.mit.edu/

79 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001, 25:402- 408.

80 Pfaffl MW. Nucleic acids: mRNA identification and quantification. In: Encyclopaedia of Analytical Science, 2nd ed., 2005, 417-426.

81 Tichopad A, Dilger M, Schwarz G, Pfaffl MW.: Standardized determination of real-time PCR efficiency from a single reaction set-up. *Nucleic Acids Res*, 2003, 31: e122.

82 Bouffaut JC, Willemart JP. Anabolic activity of trenbolone acetate alone or in association with estrogens. In: Anabolics in Animal Production (E. Meissonier, ed.), 155-179. Soregraph, Levallois, 1993.

83 Garrison JB, Kyprianou N. Novel targeting of apoptosis pathways for prostate cancer therapy. Curr *Cancer Drug Targets*, 2004 Feb;4(1):85-95

84 Baxter RC. Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *Am J Physiol Endocrinol Metab.* 2000, 278 (6): E967-E976.

85 Bechet D, Tassa A, Taillandier D. Lysosomal proteolysis in skeletal muscle. *J Biochem Cell Biol*. 2005, 37: 2098-2114.

86 Florini JR, Ewton DZ, Coolican SA. Growth hormone and the insulin-like growth factor system in myogenesis. *Endocr Rev,* 1996, 17(5): 481-517.

87 Jones JI, Clemmons DR. Insulin-Like Growth Factors and Their Binding Proteins: Biological Actions. *Endocrine Reviews,* 1995, 16(1): 3-34

88 Bartoli M, Richard I. Calpains in muscle wasting. *Int J Biochem Cell Biol.* 2005, 37 (10): 2115-2133.

89 Dargelos E, Moyen C, Dedieu S. Development of an inducible system to assess p94 (CAPN3) function in cultured muscle cells. *J Biotech*, 2002, 96: 271-279.

90 Stockholm D, Herasse M, Marchand S, Praud C, Roudaut C, Richard I, Sebille A, Beckmann JS. Calpain 3 mRNA expression in mice after denervation and during muscle regeneration. *Am J Physiol Cell Physiol*, 2001, 280(6):C1561-9.

91 Karstila T, Rechardt L, Honkaniemi J, Gustafsson JA, Wikströms AC, Karpinnen A, Pelton-Huikko M. Immunohistocytochemical localization of glucocorticoid receptor in rat skin. *Histochemistry*, 1994, 102(4):305-9.

92 Sohn KC, Jang S, Choi DK, Lee YS, Yoon TJ, Jeon EK, Kim KH, Seo YJ, Park JK, Kim CD. Effect of thioredoxin reductase 1 on glucocorticoid receptor activity in human outer root sheath cells. *Biochem Biophys Res Commun*, 2007, 11;356(11):810-5.

93 Ando Y, Yamaguchi Y, Hamada K, Yoshikawa K, Itami S. Expression of mRNA for androgen receptor, 5α-reductase and 17β-hydroxasteroid dehydrogenase in human dermal papilla cells. *Br J Dermatol*, 1999, 141:840-845.

94 Mitsui S, Ohuchi A, Hotta M, Tsuboi R, Ogawa H. Genes for a range of grwoth factors and cyclin-dependent kinase inhibitors are expressed by isolated human hair follicles. *Br J Dermatol*, 1997, 137:693-698.

95 Ohnemus U, Uenala M, Conrad F, Handjiski B, Mecklenburg L, Nakamura M, Inzunza J, Gustafsson JA, Paus R. Hair cycle control by estrogens: catagen induction via estrogen receptor (ER)-α is checked by ERβ signaling. *Endocrinology*, 2005, 146:1214-1225.

96 Randall VA, Thorton MJ, Hamada K, Messenger AG. Mechanism of androgen action in cultured dermal papilla cells derived from human hair follicle with varying resposes to androges in vivo. *J Invest Dermatol*, 1992, 98:86S-91S.

97 Winiarska A, Mandt N, Kamp H, Hossini A, Seltmann H, Zouboulis CC, Blume-Peytavi U. Effect of 5α-Dihydrotestosterone and testosterone on apoptosis in human dermal papilla cells. *Skin Pharmacol Physiol*, 2006, 19:311-321.

Acknowledgments

Especially I want to thank Prof. Dr. Dr. Heinrich H.D. Meyer who gave me the possibility to do my PhD thesis at the chair of Physiology and who supervised me during this time. I want to thank him for his trust in my work and my ideas, so that I learned to think independently and critically.

I also want to thank PD Dr. Michael W. Pfaffl, supervisor of the methodical part of my thesis, for his help with all the questions concerning PCR and for the really good working atmosphere.

Many thanks to Dr. Alês Tichopad who help me so much with the statistic and who taught me that there are always several ways to analyse results.

Thanks to Dr. Susanne E. Ulbrich who introduced me in histology and always had helpful ideas.

Special thanks go to my boyfriend Hermann who supported me during this time and tolerated my moods. Also thanks to my family who always assisted me in my decisions.

Warm thanks to my PhD colleagues and all employees at the institute who helped me with all my problems, questions and hair sample collection. Special thanks to the students Arne Christians and Sonja Englert for their help.

I am deeply grateful for the financial support of the projects by “TAP Pharmaceutics” and the “Bundesinstitut für Sportwissenschaften”.

Scientific communications

Original Publications

Reiter M, Walf VM, Christians A, Paffl MW, Meyer HHD.
Modification of mRNA expression after treatment with anabolic agents and the usefulness for gene expression-biomarkers.
Analytica Chimica Acta, 2007, 586:73-81.

M. Reiter, M.W. Pfaffl
Effects of plate position, plate type and sealing systems on real-time PCR results.
Biotechnology and Biotechnological Equipment, 2008, 22/3.

Reiter M, Lüderwald S, Pfaffl M.W, Meyer H.H.D
First steps toward a new screening method for anabolic androgenic androgens in human hair follicle.
Submitted: "Doping Journal"

Reiter M, Pfaffl M.W, Schönfelder M, Meyer H.H.D.
Gene expression in hair follicle dermal papilla cells after treatment with stanozolol.
Submitted: "Biomarkers"

M. Reiter, A. Tichopad, I. Riedmaier, M.W. Pfaffl, H.H.D. Meyer
Monitoring gene expression in muscle tissue of *macaca fascicularis* under the influence of testosterone and SARM.
Submitted: "Current Drug Discouvery Technologies"

Oral presentations

Reiter M, Walf VM, Christians A, Pfaffl MW, Meyer HHD.
Modification of mRNA expression after treatment with anabolic agents and the usefulness for gene expression-biomarkers.
5th International Symposium on hormone and veterinary drug residue analysis, Universiteit Gent, 16.04-19.04.2006, Antwerp, Belgium.

Reiter M, Walf VM, Christians A, Paffl MW, Meyer HHD.
Attempts to identify gene expression biomarkers after treatment with anabolic agents.
47. Arbeitstagung der Deutschen Veterinärmedizinischen Gesellschaft e.V., 27.09.2006, Kongresshaus Garmisch-Partenkirchen.

Posters

Reiter M, Walf VM, Paffl MW, Meyer HHD.
Effects of Anabolic Sex Hormones on Gene Expression in Bovine Liver –
Differential Gene Expression Profiling via a candidate gene approach qRT-PCR
2nd International qPCR Symposium, Technische Universität München, 05.09-09.09.2005, Freising-Weihenstephan, Germany.

Reiter M, Walf VM, Christians A, Paffl MW, Meyer HHD.
Modification of Metabolic Enzymes and their Usefulness for Biomarkers, after treatment with Anabolic Agents
5th International Symposium on hormone and veterinary drug residue analysis, Universiteit Gent, 16.04-19.04.2006, Antwerp, Belgium.

Reiter M, Walf VM, Christians A, Paffl MW, Meyer HHD.
Drug screening via modified gene expression pattern: Effects of anabolic agents on biochemical pathways
International Symposium: harmonising the knowledge about biomedical side effects of doping, Technische Universität München, 21.10.2006, München, Germany.

Reiter M, Pfaffl MW, Schönfelder M, Meyer HHD.
Doping analysis- Gene expression in hair follicle cells as new screening method?
3rd International qPCR Symposium, Technische Universität München, 26.03- 30.3.2007, Freising-Weihenstephan, Germany.

Curriculum vitae

Martina Reiter

Place and date of birth: Rosenheim (Germany), 20.01.1981

Education:

PhD thesis (Dr.rer.nat) Zentralinstitut für Ernährungs- und Lebensmittelforschung Technische Universität München, Germany Project: Modification of metabolic pathways by anabolic agents and identification of gene expression biomarkers	since 05/2005
Scientific assistant Institute of Biological Chemistry and Nutrition, Universität Hohenheim, Germany Project: Antioxidant system and vitamin A in cystic fibrosis	09/2004-04/2005
Study of sport science Justus-Liebig-Universität Giessen, Germany Minors: sport psychology, training strategies	02/2003-04/2004
Study of Nutrition Science Justus-Liebig-Universität Giessen, Germany Minors: endocrinologie, microbiologie Diploma thesis: Loss of elecrolytes during endurance exercise Diploma degree	10/1999- 04/2004
Matura (final examination at high school) Bundesrealgymnasium Kufstein, Austria	10/1991- 07/1999
Primary school Volksschule Kiefersfelden, Germany	09/1987- 07/1991

APPENDIX

Appendix I

Available online at www.sciencedirect.com
ScienceDirect
Analytica Chimica Acta 586 (2007) 73–81
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Modification of mRNA expression after treatment with anabolic agents and the usefulness for gene expression-biomarkers

Martina Reiter*, Vanessa M. Walf, Arne Christians, Michael W. Pfaffl, Heinrich H.D. Meyer

Physiology Weihenstephan, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Germany

Received 9 June 2006; received in revised form 8 September 2006; accepted 30 October 2006
Available online 7 November 2006

Abstract

With this feasibility study a first step towards a new monitoring system for hormonal treatments was done. Screening of regulation and function of anabolic sex steroids via modified gene expression of mRNA in various tissues could be a new approach to trace treatments with unknown drugs or newly combined cocktails.

In the study, uterus, liver and muscle tissue from 24 cycling heifers were taken after the animals were treated either with Melengestrol Acetate (MGA), Finaplix-H® (200 mg Trenbolone Acetate) or Ralgro® (36 mg Zeranol) for 56 days. In every treatment group always two heifers were given 1-fold, 3-fold and 10-fold doses of the standard preparation, the control group without any treatment consisted of two animals. The different tissue gene expression profiles were investigated via the candidate gene approach. Totally 57 candidate genes were selected according to their functionality by screening the actual literature and composed to functional groups: angiogenesis, apoptosis, cell cycle, endocrine factors, energy metabolism, inflammatory factors, muscle function, oncogenes, protein metabolism and transcription factors. Gene expression was measured using quantitative real-time RT-PCR (qRT-PCR) technology.

From 24 tested candidate genes in the liver, 17 showed a significant regulation. Eight genes were influenced by MGA, 9 by Finaplix-H®, and 4 by Ralgro®. For the muscle tissue 19 genes were tested with the result that in the neck muscle 11 genes were regulated and in the hind limb muscle 8 genes. In the neck 5 genes were affected by MGA, 6 by Finaplix-H® and 3 by Ralgro®. Only 2 genes were influenced by MGA in the hind limb muscle. Finaplix-H® affected 6 and Ralgro® 4 genes. In the uterus 29 target genes were tested and 13 were significantly influenced by the anabolic sex steroids. Under Finaplix-H® treatment eight target genes were regulated and Ralgro® and MGA showed a significant regulation in four target genes.

The highest gene expression changes under anabolic treatment were observed in the uterus. The analyzed genes showed significant regulations but further studies, testing different animal husbandry conditions will be needed to identify meaningful expression patterns for the different tissues.

With the investigation of the regulation and possible function of anabolic sex steroids via gene expression, a preparatory work for the development of an expression pattern for drug screening was made.

Keywords: Anabolic agents; Expression profiling; Candidate gene approach; Melengestrol acetat; Trenbolone acetat; Zeranol; Real-time quantitative reverse transcriptase polymerase chain reaction

1. Introduction

In Canada, the USA and other countries, the use of xenobiotic, anabolic sex steroids in farm animals is licensed. Melengestrol acetate (MGA), trenbolone acetate (TBA) and zeranol are used to induce better growth rates, reduce fat thickness, and receive leaner animals with more meat [4,18,24,30]. In the EU the registration and application of anabolic hormones is forbidden because of potential health risks for the consumers [13].

MGA is taken to improve feed efficiency and growth while suppressing the ovulation and inducing endogenous estradiol [15,29,37]. TBA is active after hydrolysis to trenbolone-17beta. It also acts as anti-glucocorticoid and provokes synthesis of myofibrillar proteins in muscle. Trenbolone binds to the androgen receptor with a higher affinity than dihydrotestosterone, to the progestin receptor and even to the glucocorticoid receptor

Abbreviations: AM, arithmetic mean; bp, base pairs; CP, crossing point; DNA, desoxyribonucleic acid; for, forward primer; RIN, RNA integrity number; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; rev, reverse primer; RG, reference gene; mRNA, messenger ribonucleic acid; CG, candidate gene

* Corresponding author. Tel.: +49 8161713867.
E-mail address: martina.reiter@wzw.tum.de (M. Reiter).

0003-2670/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2006.10.049

[28,40,41]. Zeranol is derived from the mycotoxin zearalenone and provokes higher absorption rate on the regular nutrition level in heifers and improves growth rates of cattle [22].

Applying the anabolic agents, changes in the biochemical pathways are notified in 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 feasibility study was to show how anabolic sex steroids, like MGA, TBA and zeranol act in different bovine tissues via mRNA expression of different target genes. The discovered, regulated target genes could then function as biomarkers for the design of characteristic expression patterns of anabolic agent groups. Gene expression from the following candidate gene groups were investigated: angiogenesis, apoptosis, cell cycle, endocrine factors, energy metabolism, inflammatory factors, muscle function, oncogenes, protein metabolism and transcription factors.

2. Experimental

2.1. Animal experiment

Groups of six Holstein–Friesian heifers were treated either orally with 0.5 mg MGA or with the implant preparations of Finaplix-H® (200 mg Trenbolone Acetate; Hoechst Roussel Vet, Sommerville, USA) or Ralgro® (36 mg Zeranol; Mallinckrodt Veterinary Inc., Mundelein, USA). According to the manufacturers instruction, the implants were administered to the middle third of the pinna of the right ear. In every treatment group always two heifers were given 1-fold, 3-fold and 10-fold doses of preparation, the control group without any treatment consisted of two animals per group. The heifers were aged between 12 and 16 month with a mean weight of 320 ± 57 kg [12,15,24,36].

2.2. Sample collection

After 56 days treatment all heifers were slaughtered, tissue samples were taken and stored at $-80\,^{\circ}C$ until total RNA extraction.

2.3. Total RNA extraction

200 mg of frozen tissue was homogenized in 4 M guanidinium thiocyanate buffer to destroy RNase activity. For the extraction of the RNA an AGS clean protocol was used (AGS RNA-Clean, AGS, Heidelberg, Germany). The principal of this protocol was phenol/chloroform extraction for total RNA. To quantify the amount of total RNA extracted, absorbance was measured with the photometer (Eppendorf Biophotometer, Hamburg, Germany) for each sample. RNA purity was calculated with the 260/280 absorbance ratio. All working solutions were diluted to a concentration of 10 ng RNA μL^{-1}.

2.4. Analysis of RNA quality

RNA integrity and quality control was performed with 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 [13,31].

2.5. Selection of target genes

Target genes, factors and enzymes were selected if supposed to play an important role in the anabolic pathways of the analyzed tissues. Following candidate genes were chosen by screening the respective literature.

Endocrine factors consist of the different hormone receptors like the androgen receptor (AR), the estrogen receptors (ERα, ERβ), the growth hormone receptor (GHR), the prolactin receptor (PRLR), the insulin receptors (IRα, IRβ), and the glucocorticoid receptor (GRα). The insulin-like growth factor (IGF-1), its receptor (IGF-1-R) and its binding protein (IGF-BP3) also belong to this group [28].

The investigation of all factors, which are included in proteolysis and protein synthesis is of interest because of the anabolic function. To this protein metabolism group belongs cathepsin L (CTSL), cathepsin B (CTSB) and calpastatin (CAST), calpain (CAPN3) and tyrosin-amino-transferase (TAT) [21,23,32,34,42,43,45].

The group of muscle function includes the fiber proteins actin and its isoforms (ACTA1, β-actin) and myosin with its isoforms (MYHC-2x) [38,47,48].

In adipocytes, anabolic hormones induce lipolyis, what results in several candidate enzymes such as enoyl-coA-hydratase (enoylCoA) or acyl-coA-dehydrogenase very long chain (ACADvl) [39].

The enzyme creatinkinase (CK) plays an important role in the regeneration of ATP. Glycolysis is a major pathway in the muscle and hexokinase (HK) plus lactate-dehydrogenase (LDH) are involved. Together with aconitase, key enzyme of the citric acid cycle, these genes are summarized as energy metabolism group [49].

Cyclin-dependend kinase (CDK2), cyclin A and cyclin D1 are factors of the cell cycle regulation and induce muscle growth [3,25]. Myostatin, a member of the TGF-β family is an antagonist of this regulation [6].

RNA expression is influenced by different transcription factors, like c-fos protein (c-fos) or c-jun protein (c-jun) [5].

It is supposed that anabolic agents also affect angiogenesis. The vascular endothelial growth factors (VEGFs) and their receptor (FLK-1), the fibroblast growth factors (FGFs), angiopoetin (Angpt1, Angpt2), the matrix metalloproteinases (MMP1,2,14) and their inhibitors (TIMP1,2) are members of this functional group.

The influence of anabolic agents on pro-inflammatory response and reactions is still unclear. Tumor necrosis factor-α

(TNF-α), interleukin-1β (IL1-β) and cyclo-oxygenase 2 (Cox2) belong to this group.

Factors which are involved in cell death regulation like Fas-L, Caspase3 and bcl-xl were summarized in the apoptosis group [17,19].

Hormones are associated with certain kinds of cancer, especially in the liver. Factors that are supposed to play a role in hepatocellular carcinoma are p53 (tumor suppressor protein) and v-myb and were summarized as oncogenes [7,11,26,33,50].

All selected target genes were composed to functional groups in Table 1.

2.6. Primer design and primer testing

All primers were designed using published nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Primer design and optimization was done with HUSAR program at DKFZ (http://www.genius.embnet.dkfz-heidelberg.de/menu/w2h/w2hdkfz/) with regard to primer dimer formation, self-priming formation and primer annealing temperature (60 °C). Primer sequences of regulated genes are summarized in Table 1. New designed primers were ordered and synthesized at MWG Biotech (Ebersberg, Germany). Primer testing was performed with 4 random samples and a negative control (RNAse free water) for each primer set.

2.7. One-step quantitative RT-PCR

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 manufacture. The master mix was prepared as follows: For one sample 5 μL 2× SYBR Green Reaction Mix, 0.5 μL forward primer (10 pmol μL^{-1}), 0.5 μL reverse primer (10 pmol μL^{-1}) and 0.2 μL SYBR Green One-Step Enzyme Mix is used. 6.2 μL of the master mix was filled in the special 25 μL tubes and 1 ng μL^{-1} total RNA in 3.8 μL volume was added. Tubes were closed, placed into the Rotor-Gene 3000 and Analysis Software V5.0 was started (Corbett Life Science, Sydney, Australia). The following RT-PCR protocol was used: hold step I (55 °C, 10 min), denaturation step (95 °C, 5 min), cycling program (95 °C, 15 s; 60 °C, 30 s; 68 °C, 20 s) hold step II (40 °C, 20 s) and melting curve analysis. Crossing Points (CP) and melting curves were acquired by using the "*comparative quantitation*" and "*melting curve*" program of the Rotor-Gene analysis software.

2.8. Agarose gel electrophoresis

All real-time RT-PCR products from primer testing were applied on a high resolution agarose gel and electrophoresis was conducted at 90 V for 30 min. Agarose gel was then photographed under UV-excitation to enable analysis of fragment size, band intensity and integrity (gel data not shown).

2.9. Data analysis and statistics

Quantitative real time PCR data were processed applying relative quantification method using the ΔΔCP-method ($2^{\Delta\Delta CP}$) [27]. Expression changes are shown as relative up- or down-regulation compared to three internal reference genes. For normalization of target gene expression the arithmetic mean (AM) of the following non regulated reference genes (RG) were taken: (RG1) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (RG2) β-actin and (RG3) ubiquitin. For every sample gene expression of all three RG were analyzed and the mean value served as reference gene index. The measurement of the reference genes showed a significant up-regulation of GAPDH in muscle tissue and of β-actin under the influence of MGA the uterus tissue. Only the left two reference genes (ubiquitin and β-actin in muscle; ubiquitin and GAPDH in the uterus) were taken for normalization.

In Excel (Microsoft, USA) the regression of ΔΔCP on treatment dose was calculated in a linear regression model for MGA, Finaplix-H® and Ralgro® over all target genes. Significance minimum was $P < 0.05$.

In the first step of the ΔΔCP-method the crossing point of the AM of the reference genes (RG) was subtracted from the crossing point (CP) of the candidate gene. From the so calculated ΔCPs the mean value of the repeat determination was taken to get the ΔCP of the treatment. From this ΔCP the mean value of ΔCP of the non treated control was subtracted. The result is the ΔΔCP value. In this calculation model the optimal PCR efficiency, representing a DNA doubling after each cycle ($E = 2$), is assumed. Therefore the expression ratio of a candidate gene compared to the control treatment of the analysis is expected as $2^{\Delta\Delta CP}$.

$$\Delta CP = CP_{(AM)} - CP_{(candidate\ gene)}$$

$$\Delta CP_{(treatment)} = \text{mean } \Delta CP \text{ value of repeat determination}$$

$$\Delta CP_{(control)} = \text{mean } \Delta CP \text{ value of control animals}$$

$$\Delta\Delta CP = \Delta CP_{(treatment)} - \Delta CP_{(control)}$$

3. Results

3.1. RNA integrity

In the liver, muscle and uterus the mean ± S.D. RIN values were 7.72 ± 0.69, 7.57 ± 1.24, and 7.26 ± 1.81, respectively (box plots shown in Fig. 1).

3.2. Primer testing and gel electrophoresis

Primer pairs of 57 genes were used for qRT-PCR analysis to get single peaks and uniform melting curves, as well as a specific single bands in agarose gel electrophoresis.

Table 1
Primer sequences of analyzed target genes with primer length (bp) and analyzed tissue (L = liver, M = muscle, U = uterus, for = forward primer, rev = reverse primer)

Group	Primer	5′–3′	Size (bp)	Tissue
Angiogenesis	Angpt 1 for	TTC CTC GCT GCT ATT CTG ACT	194	U
	Angpt 1 rev	TGA CAG CAC TCT CAT GCT GTC		
	Angpt2 for	AAT TCA GT CTC CAA AAG CAG C	234	U
	Angpt2 rev	TCC ACC CGT TTC CAT GTC		
	FGF2 for	AGC CTT GCA ACT CTG CTT GT	210	U
	FGF2 rev	CGA ATT CAG ATC CCT CCT GA		
	FGF7 for	CTG CCA AGT TTG CTC TAC AG	294	U
	FGF7 rev	TCC AAC TGC CAG GGT CCT GAT		
	FLK-1 for	GCT TCT ACC AGG ACA CTG ACA T	144	U
	FLK-1 rev	AAC ACG GAA TCA CCA CCA CAG TT		
	MMP1 for	GAG GAG ACG CTC ATT TTG ATG	235	U
	MMP1 for	ACT GGC TGA GTG GGA TTT TG		
	MMP2 for	CCC AGA CAG TGG ATG ATG C	237	U
	MMP2 rev	TTG TCC TTC TCC CAG GGT C		
	MMP14 for	ACT TGG AAG GGG GAC ACC	232	U
	MMP14 for	AGG GGG CAT CTT AGT GGC		
	TIMP1 for	CAT CTA CAC CCC TGC CAT G	231	U
	TIMP1 rev	CAG GGG ATG GAT GAG CAG		
	TIMP2 for	GGG TCT CGC TGG ACA TTG	255	U
	TIMP2 rev	TTG ATG TTC TTC TCC GTG ACC		
	VEGF all for	CCG TCC CAT TGA GAC CCT G		U
	VEGF120 rev	CGG CTT GTC ACA ATT TTT CTT GTC	280	
	VEGF164 rev	GCC CAC AGG GAT TTT CTT GC	278	
	VEGF188 rev	TGC CCC TTT CCC TTT CCT C	296	
	VEGF for	GGT GGA CAT CTT CCA GGA GTA	177	M
	VEGF rev	CTA TGT GCT GGC TTT GGT GAG		
Apoptosis	bcl-xl for	GGC ATT CAG CGA CCT GAC	203	L, U
	bcl-xl rev	CCA TCC AAG TTG CGA TCC		
	Casp3 for	GCA ACG TTT CTA AAG AAG ACC ATA G	64	U
	Casp3 rev	CCA TGG CTT AGA AGC ACA CAA ATA A		
	Fas-L for	CAT CTT TGG AGA AGC AAA TAG	205	L, U
	Fas-L rev	GGA ATA CAC AAA ATA CAG CCC		
Cell cycle	CDK2 for	CTC ATC GAG TCC TGC ACC G	183	M
	CDK2 rev	GTA GTA TTT GCA GCC CAG AAG G		
	Cyclin A for	GCT ATC CTC GTG GAC TGG TTA	159	M
	Cyclin A rev	AAG CAT AGC AGC AGT GCC CA		
	Cyclin D1 for	GAA GAT GAA GGA GAC CAT CCC	190	M
	Cyclin D1 rev	TTG TTC TCC TCT GCC ACT GG		
	Myostatin for	GCC TGT CAA GAC TCC TGC GA	173	M
	Myostatin rev	TGT TTG AGC CAG TTC TGC AAC AC		
Endocrine factors	AR for	CCT GGT TTT CAA TGA GTA ACC GCA TG	172	M, U
	AR rev	TTG ATT TTT CAG CCC ATC CAC TGG A		
	ER alpha for	AGG GAA GCT CCT ATT TGC TCC	234	M, U
	ER alpha rev	CGG TGG ATG TGG TCC TTC TCT		
	ERbeta for	GAG ATA TTC TTT GTG TTG GAG TTT	242	U
	ERbeta rev	CTT CGT GGA GCT CAG CCT GT		
	GHR for	CCA GTT TCC ATG GTT CTT AAT TAT	136	L, U
	GHR rev	TTC CTT TAA TCT TTG GAA CTG G		
	GR alpha for	TTC GAA GAA AAA ACT GCC CAG C	190	L, M
	GR alpha rev	CAG TGT TGG GGT GAG TTG TG		
	IGF1 for	CCA AAG GCC AGA CCT ACT TG	180	L, M, U
	IGF1 rev	TCC TCA GAT CAC AGC TCC GA		
	IGF1-R for	TTA AAA TGG CCA GAA CCT GAG	314	L, U
	IGF1-R rev	ATT ATA ACC AAG CCT CCC AC		
	IGF-BP3 for	ACA GAC ACC CAG AAC TTC TCC T	202	L, M, U
	IGF-BP3 rev	AGA AAC CCC GCT TCC TGC C		
	IR alpha for	TCC TCA AGG AGC TGG AGG AGT	89	L
	IR alpha rev	TTT CCT CGA AGG CCT GGG GAT		
	IR beta for	TCC TCA AGG AGC TGG AGG AGT	111	L
	IR beta rev	TAG CGT CCT CGG CAA CAG G		
	PRLR for	AAG GAA GGA GAA ACA CTC ATC CA	203	L
	PRLR rev	AGG TTT GCA GGA GGC TCT G		

Table 1 (*Continued*)

Group	Primer	5′–3′	Size (bp)	Tissue
Energy metabolism	ACAD vl for	ACT TTG ACG GAG TAC GGG TG	198	L
	ACAD vl rev	CAA AGT TGT GAA TTT TCT CCC C		
	Aconitase for	CAT CCG AGT TGG TCT GAT TG	188	U
	Aconitase rev	ACA TCC CTC AGG ATC TGT G		
	CK for	ATG ACA GAG CAG GAG CAG CA	183	M
	CK rev	ATG GAG ATG ACT CGG AGG TG		
	Enoyl-CoA for	GCT GCT GTC AAT GGC TAT GC	202	L
	Enoyl-CoA rev	ACC AGT GAG GAC CAT CTC CA		
	HK for	CAA GAC GCA CCC ACA GTA TCC	211	M, U
	HK rev	TCA CCT CCA GCA GCA TTT CCT T		
	LDH for	GTG GCT TGG AAG ATA AGT GG	155	M, U
	LDH rev	ACT AGA GTC ACC ATG CTC C		
Inflammatory factors	Cox2 for	CCT GAT GAC TGC CCA ACA C	162	L
	Cox2 rev	AAA TTG ATG GGT GAA GTG CTG G		
	IL1 beta for	TTC TCT CCA GCC AAC CTT CAT T	198	L
	IL1 beta rev	ATC TGC AGC TGG ATG TTT CCA T		
	TNF alpha for	TAA CAA GCC GGT AGC CCA CG	256	L
	TNF alpha rev	GCA AGG GCT CTT GAT GGC AGA		
Muscle function	ACTA1 for	TAT TGT GCT CGA CTC CGG CGA	160	M, U
	ACTA1 rev	GTC ACG AAG GAG TAG CCA CG		
	MYHC-2x for	GAT CAA TGC TGA GCT GAC GG	173	M, U
	MYHC-2x rev	CAA TGG TTT CAT CCA GAC CTG C		
Oncogenes	p53 for	GAC TTC TCT CGG CGC CTA CC	191	L, U
	p53 rev	TGG TGC ACT CAG AGT CGA TC		
	v-myb for	TCA CGT CCC ATA TCC TGT AGC	170	L
	v-myb rev	CCT GTC CTT TGA GTT CGT TCT CA		
Protein metabolism	CAPN 3 for	GAC TGG AGC TAT GTG GAC AAG	179	M
	CAPN 3 rev	GTC CAA GTC TGA AGC TTG TCG		
	CAST for	GAT CAG AAG TGC TGC TCC A	206	L
	CAST rev	GGA CTG TTT CCT CAT CTT ACC		
	CTSB for	GAT CTG CAT CCA CAG CAA	192	L, M, U
	CTSB for	ATG GAG TAC GGT CTG CAA CC		
	CTSL for	CAC TGG TGC TCT TGA AGG ACA	183	L, M, U
	CTSL rev	TAA GAT TCC TCT GAG TCC AGG C		
	TAT for	ACC CTT GTG GGT CAG TGT TC	167	M
	TAT rev	ACA GGA TGG GGA CTT TGC TG		
Transcription factors	c-Fos for	GCT CCA GGC GGA GAC AGA	302	L
	c-Fos rev	AGG GTG AAG GCC TCC TCA GA		
	c-jun for	TCA ACG CCT CGT TCC TCC	278	L
	c-jun rev	CTC ATC TGT CAC GTT CTT GGG GCA		
Reference genes	beta Actin for	AAC TCC ATC ATG AAG TGT GAC	202	L, M
	beta Actin rev	GAT CCA CAT CTG CTG GAA GG		
	GAPDH for	GTC TTC ACT ACC ATG GAG AAG G	197	L, U
	GAPDH rev	TCA TGG ATG ACC TTG GCC CAG		
	Ubiquitin 3 for	AGA TCC AGG ATA AGG GAA GGC AT	198	L, M, U
	Ubiquitin 3 rev	GCT CCA CCT CCA GGG TGA T		
Others	CyP1A1 for	CCG ACC TCT ACA GCT TCA C	181	L
	CyP1A1 rev	GCC TCC TTG TTC ACA TGC TC		
	CALR for	TTG ACA ACA GCC AGG TGG AG	192	L
	CALR rev	CAG GTT TCT TAG CAT CAG GGT C		

3.3. Gene regulation in liver

Seventeen of the 24 tested target genes were significantly regulated in liver tissue. Under MGA-treatment, 6 genes were up- and 2 down-regulated. Finaplix-H® induced in nine genes an up-regulation. Under Ralgro® 4 genes were up-regulated. The increases or decreases of cycle number are shown in Table 2.

3.4. Gene regulation in muscles

Of the 19 measured target genes, 11 were significantly regulated in the neck muscle and 8 in the hind limb muscle. MGA induced an up-regulation of five genes in the neck muscle. One gene was up-regulated and five were down-regulated under Finaplix-H®. Ralgro® induced an up-regulation in two and a down-regulation in one gene. In the hind limb muscle MGA

Table 2
Expression changes in liver

Group	Factor	Treatment	ΔΔCP	Significance
Energy metabolism	EnoylCoA	MGA		
		FINAPLIX	**0.5**	**
		RALGRO		
	ACADvl	MGA		
		FINAPLIX	**0.6**	**
		RALGRO		
Apoptosis	bcl-xl	MGA		*
		FINAPLIX	**0.7**	***
		RALGRO		
	FasL	MGA		
		FINAPLIX	**0.4**	*
		RALGRO	**1.4**	***
Endocrine factors	IGF1	MGA		
		FINAPLIX		
		RALGRO	**1.4**	**
	IR alpha	MGA		
		FINAPLIX	**0.9**	*
		RALGRO	**0.1**	*
	IR beta	MGA	**0.4**	*
		FINAPLIX		
		RALGRO		
	PRLR	MGA	**0.8**	**
		FINAPLIX		
		RALGRO		
	GR alpha	MGA		
		FINAPLIX		
		RALGRO	**0.9**	***
	GHR	MGA	**0.9**	*
		FINAPLIX	**1.0**	**
		RALGRO		
Inflammatory factors	IL1 beta	MGA		
		FINAPLIX	**0.8**	**
		RALGRO		
	Cox2	MGA	**−1.3**	*
		FINAPLIX		
		RALGRO		
Protein metabolsim	CAST	MGA	**0.5**	*
		FINAPLIX		
		RALGRO		
	CTSB	MGA		
		FINAPLIX	**1.0**	***
		RALGRO		
	CTSL	MGA	**1.2**	**
		FINAPLIX		
		RALGRO		
Oncogene factors	p53	MGA	**−0.7**	*
		FINAPLIX		
		RALGRO		
	v-myb	MGA	**1.8**	**
		FINAPLIX	**1.5**	*
		RALGRO		
Transcription factors	c-fos	MGA	**1.2**	*
		FINAPLIX		
		RALGRO		

Linear regression coefficients of dosages regressed on ΔΔCP with corresponding significance level. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

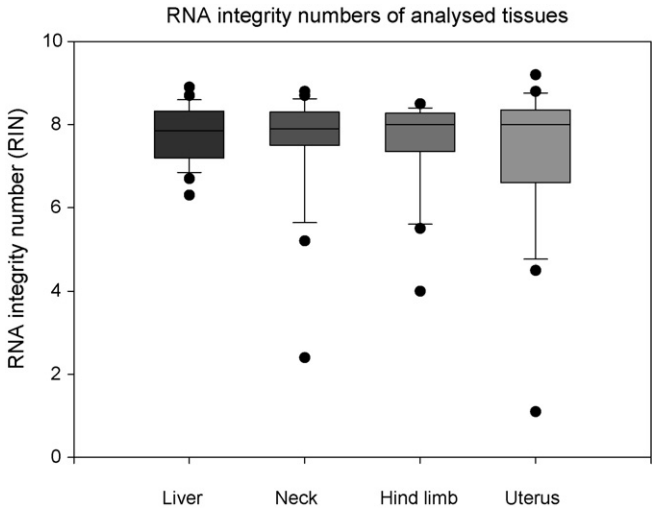


Fig. 1. Results of bioanalyzer based RNA integrity rating across all treatments and tissue samples.

and Finaplix-H® treatment induced an up-regulation in two genes and four genes were additionally down-regulated under Finaplix-H®. Ralgro® could up-regulate three genes and one gene was down-regulated. All regulations are summarized in Tables 3 and 4.

3.5. Gene regulation in uterus

Significant gene expression in uterus tissue was estimated in 13 of the 29 tested target genes. Under the treatment of MGA an up-regulation of three genes and a down-regulation of one gene was found. Finaplix-H® provoked an up-regulation of six genes, two genes were down-regulated under this treatment. Under the influence of Ralgro® only one gene was up-regulated, three genes were down-regulated. A summary of the regulations is shown in Table 5.

4. Discussion

The effects of three anabolic steroids were examined in four different tissues via mRNA gene expression of specific target genes with the aim to investigate metabolic pathways and to find useful biomarkers.

The expression of all target genes was determined by qRT-PCR, a technique, which allows a rapid and reliable quantification of ribonucleic acids. By analyzing the CP of each gene in each sample, very precise and exact results of the amount of analyzed nucleic acids could be calculated [8].

The outcome of gene expression results is strongly influenced by RNA quality. To exclude the possibility of degraded RNA, all samples were measured using the lab-on-a-chip technology of Agilent Bioanalyzer. The samples were analyzed by capillary electrophoresis in a channel network, based on a nano chip. The good results for RNA quality showed that RNA is stable and intact over years by adequate storage [13,31].

The target genes showed very different regulations in the functional groups and individual tissues and the number of analyzed genes was yet too less to have precise conclusions of the

Table 3
Expression changes in neck muscle. Linear regression coefficients of dosages regressed on ΔΔCP with corresponding significance level

Group	Factor	Treatment	ΔΔCP	Significance
Muscle function	ACTA1	MGA	**0.6**	*
		FINAPLIX	**−0.3**	*
		RALGRO		
	MYHC-2x	MGA		
		FINAPLIX	**−0.9**	*
		RALGRO		
Cell cycle	CDK2	MGA	**0.8**	*
		FINAPLIX		
		RALGRO		
	Cyclin A	MGA		
		FINAPLIX		
		RALGRO	**1.7**	*
	Myostatin	MGA		
		FINAPLIX	**−1.6**	*
		RALGRO		
Endocrine factors	ER alpha	MGA	**0.7**	**
		FINAPLIX		
		RALGRO	**−0.5**	*
	GR alpha	MGA	**0.5**	*
		FINAPLIX	**−1.0**	*
		RALGRO		
	IGF-1	MGA		
		FINAPLIX		
		RALGRO	**1.2**	*
	IGF-BP3	MGA	**1.4**	*
		FINAPLIX		
		RALGRO		
Energy metabolism	HK	MGA		
		FINAPLIX	**0.3**	*
		RALGRO		
	LDH	MGA		
		FINAPLIX	**−0.9**	*
		RALGRO		

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

Table 4
Expression changes in hind limb muscle

Group	Factor	Treatment	ΔΔCP	Significance
Muscle function	MYHC-2x	MGA		
		FINAPLIX	**−1.0**	*
		RALGRO		
Protein metabolism	CTSL	MGA		
		FINAPLIX	**0.8**	*
		RALGRO	**0.9**	***
Cell cyclus	Cyclin A	MGA	**1.5**	**
		FINAPLIX		
		RALGRO		
Endocrine factors	AR	MGA		
		FINAPLIX	**−0.6**	*
		RALGRO		
	GR alpha	MGA	**0.9**	**
		FINAPLIX	**−1.1**	***
		RALGRO	**0.6**	*
	IGF-1	MGA		
		FINAPLIX		
		RALGRO	**1.0**	**
Energy metabolism	HK	MGA		
		FINAPLIX	**0.4**	*
		RALGRO	**−0.4**	*
	LDH	MGA		
		FINAPLIX	**−1.4**	***
		RALGRO		

Linear regression coefficients of dosages regressed on ΔΔCP with corresponding significance level. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

specific pathways. The highest gene expression differences were recognized in the uterus. The changes in mRNA levels can be expected because this primary sexual organ is fundamentally influenced by sexual hormones. Lower gene expression differences, but still significant, could be shown in liver and the two muscles.

Although, at this stage the interpretation of the results seems to be very difficult, but the first physiologic coherences and potential biomarkers could be identified.

To discover the influence of the anabolic steroids on specific metabolic pathways, the candidate gene expressions in the functional groups under the influence of a specific anabolic agents were analyzed with the following results.

4.1. Potential biomarker for MGA induced expression pattern

First biomarkers for MGA treatment were found in the uterus, in the angiogenesis group with metalloproteinase MMP2 (gelatinase A) that showed a significant down-regulation parallel to a significant up-regulation of its inhibitor TIMP2. The collagenase MMP2 depends on the extracellular matrix destruction-related molecules, like collagens and laminin. Especially MMP2 plays an important role in cell migration, inflammation and metastasis and the use of synthetic inhibitors of MMPs to prevent disease progression, is an attractive therapeutic strategy [16,35].

In the liver MGA influenced the candidate gene expression in the oncogene group. Studies proofed that tumor suppressor protein p53 shows abnormalities in tumor cells [7,50]. The significant down-regulation of p53 in this study could go on line with this results. Additionally v-myc as oncogene was up-regulated what could mirror a possible effect of MGA on the development of hepatocellular carcinoma [44].

4.2. Potential biomarker for trenbolone acetate induced expression pattern

Apoptosis is a regulated process leading to programmed cell death, induced by a variety of factors. The central effector in the cascade is Caspase 3, which can be activated via an extrinsic and an intrinsic pathway [10]. Fas-L is one of the stimulating factors of the extrinsic pathway. In our study an increase of Fas-L and Caspase 3 could be seen in the uterus under the influence of TBA. In the liver Fas-L was also increased and bcl-xl showed an up regulation under the same treatment. It can be supposed, that TBA induced the extrinsic pathway of apoptosis and not the intrinsic, but once again further factors have to be analyzed to confirm this hypothesis.

Table 5
Expression changes in uterus

Group	Factor	Treatment	ΔΔCP	Significance
Muscle function	ACTA1	MGA		
		FINAPLIX		
		RALGRO	**−1.1**	*
Protein metabolism	CTSL	MGA		
		FINAPLIX	**1,4**	**
		RALGRO		
Angiogenese	MMP2	MGA	**−1.5**	*
		FINAPLIX		
		RALGRO		
	TIMP2	MGA	**2.2**	**
		FINAPLIX		
		RALGRO		
	Angpt 2	MGA		
		FINAPLIX	**−6.1**	**
		RALGRO		
Endocrine factors	AR	MGA		
		FINAPLIX	**1.1**	*
		RALGRO		
	ER beta	MGA		
		FINAPLIX	**−3.8**	***
		RALGRO		
	IGF-BP3	MGA		
		FINAPLIX	**2.5**	**
		RALGRO		
Energy metabolism	HK	MGA		
		FINAPLIX		
		RALGRO	**−6.6**	***
	Aconitase	MGA	**3.0**	*
		FINAPLIX	**0.9**	*
		RALGRO		
Apoptosis	Fas-L	MGA	**1.2**	*
		FINAPLIX	**2.4**	*
		RALGRO	**1.2**	*
	bcl-xl	MGA		
		FINAPLIX		
		RALGRO	**−0.9**	*
	Caspase 3	MGA		
		FINAPLEX	**1.6**	*
		RALGRO		

Linear regression coefficients of dosages regressed on ΔΔCP with corresponding significance level. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

The decrease of muscle protein expression (ACTA1, MYHC-2x) in muscle and the uterus under the influence of TBA was not expected because of the supposed anabolic effect. Anabolic agents are expected to retain nitrogen so that it is additionally available for muscle protein synthesis, but only under the treatment of the gestagen MGA an increase of α-actin was noticed is the muscle. A possible explanation might be our analysis of iso-forms in qRT-PCR because various α-actin and myosin isoforms are abundant (γ-actin: smooth muscle, MYHC2a), so that these factors could be affected by anabolic agents.

In the muscle the candidate genes of the energy metabolism group, HK and LDH showed an inverse regulation under the treatment of TBA. HK was up-regulated, LDH down-regulated and in the uterus aconitase was up-regulated what would point out an increased glycolysis and citric acid cycle without an anaerob utilization of glycose. This result is approved by the influence of TBA on EnoylCoA and ACADvl in liver. Both genes were significantly up-regulated and confirm the lypolysis stimulation of anabolic steroids.

CTSL, factor of the protein metabolism was increased in all tissues under all three treatments. CTSB was only increased in liver under the influence of TBA.

In the kidney of rats, an increased of CTSB under estradiol treatment was found, with the inverse effect under testosterone treatment [23]. Studies with CTSL knockout mice have shown, that it is involved in the epithelial cell differentiation in the skin hair, hair follicles and the bone [32,46]. The increasing effect on proteolytic factors was not expected but other studies suppose that CTSL, CTSB and calpains not directly influence the protein decomposition but rather function as regulating factors [21,34].

All these specified candidate genes can be seen as first biomarkers for a TBA screening pattern.

4.3. Potential biomarkers for zeranol expression pattern

Many studies have shown that IGF1 plays an important role in muscle development and growth. Mouse lines lacking IGF1 were characterized by underdevelopment of muscle tissue [2]. The increase of IGF1 and **IRα** in liver and IGF1 in muscle showed the stimulating influence of zeranol on these endocrine factors, approved in previous studies [1,9,14,20].

Also zeranol showed an influence on the gene expression in the apoptosis group. In uterus and liver Fas-L was up-regulated but bcl-xl showed a down-regulation in uterus. Further genes of the apoptosis pathway have to be analyzed to get a clear result of zeranol regulation in apoptosis but anyhow these candidate genes can be taken as first biomarker for a zeranol screening pattern.

Not all functional groups and gene regulations were mentioned because until now the coherence between these candidate genes would be a guess. More candidate genes depending to these groups have to be analyzed to avoid speculations of possible pathways and regulations.

With regard to possible biomarkers, the uterus seems to be the most adequate tissue for screening patterns because it showed the highest expression coefficients.

As MGA-biomarker MMP2, TIMP2, p53 and v-myb could be considered. A screening pattern for TBA could include Fas-L, Caspase3, bcl-xl, ACTA1, MYHC-2x, HK, LDH, Aconitase, EnoylCoA, ACADvl, CTSB and CTSL.

Biomarker for a zeranol pattern would be Fas-L, bcl-xl, IGF1 and IRα.

Also the candidate genes of the endocrine group have high potential to be included in such patterns but further analysis are needed to get a clearer insight of their regulation under anabolic treatment.

5. Conclusion

This feasibility study clearly showed that the analysis of gene expression via qRT-PCR is a new and promising method to identify effects of anabolic agents in animal tissue. Especially the

uterus with the highest expression differences, seems to be a promising tissue.

It has to be pointed out that the study conditions like the breed, age and nutrition of the animals, may affect in the expression as well, so that additional experiments are required to find stable biomarkers and metabolic pathways that are unaffected of surrounding conditions.

The ambitions for the future should be the identification of specific gene patterns for groups of anabolic agents in order that abuses can be uncovered.

References

[1] G.R. Adams, Br. J. Sports Med. 34 (6) (2000) 412–413.
[2] G.R. Adams, Invited Review, J. Appl. Physiol. 93 (3) (2002) 1159–1167.
[3] B. Alberts, D. Bray, J. Lewis, M. Raff, M., K. Roberts, J.D. Watson, in: Molekularbiologie der Zelle; 3. Auflage, 1. korr. Nachdruck (VCH), 1997.
[4] J.K. Apple, M.E. Dikema, D.D. Simms, G. Kuhl, J. Anim. Sci. 69 (1991) 4437–4448.
[5] M. Beato, Cell 56 (1989) 335–344.
[6] S. Bogdanovich, T.O. Krag, E.R. Barton, L.D. Morris, L.A. Whittemore, R.S. Ahima, T.S. Khurana, Nature 420 (2002) 418–421.
[7] D.G. Brachman, D.E. Halahan, M.A. Beckett, D.W. Yandell, R.R. Weichselbaum, Cancer Res. 51 (1991) 6393–6396.
[8] S.A. Bustin, A–Z of Quantitative PCR. IUL Biotechnology Series, 2004.
[9] S.A. Coolican, D.S. Samuel, D.Z. Ewton, F.J. McWade, J.R. Florini, J. Biol. Chem. 272 (10) (1997) 6653–6662.
[10] Cell Signaling Technology, Catalog and technical reference, 2005–2006.
[11] C.N. DïArville, P.J. Johnson, J. Steroid Biochem. Mol. Biol. 37 (6) (1990) 1007–1012.
[12] A. Daxenberger, D. Ibarreta, H.H.D. Meyer, Hum. Reprod. Update 7 (3) (2001) 340–355.
[13] S. Fleige, M.W. Pfaffl, Mol. A Med. 27 (2006) 126–139.
[14] D.A. Fryburg, L.A. Jahn, S.A. Hill, D.M. Oliveras, E.J. Barrett, J. Clin. Invest. 96 (4) (1995) 1722–1729.
[15] M. Hageleit, A. Daxenberger, W.D. Kraetzl, A. Kettler, H.H.D. Meyer, AMPIS 108 (2000) 847–854.
[16] K. Hashizume, T. Takahashi, M. Shimizu, J. Todoroki, A. Shimada, M. Hirata, T. Sato, A. Ito, J. Reprod. Developm. 49 (1) (2003) 45–53.
[17] J.W. Ho, J.Z. Song, Y.K. Leung, Curr. Med. Anti-Canc. Agents 5 (2) (2005) 131–135.
[18] D.W. Hunt, D.M. Henricks, G.C. Skelly, L.W. Grimes, J. Anim. Sci. 69 (1991) 2452–2462.
[19] N. Ishiguro, T. Ohzono, T. Shingawa, M. Horiuchi, M. Shingawa, J. Biol. Chem. 269 (43) (1994) 26822–26829.
[20] B.J. Johnson, M.R. Hathaway, P.T. Anderson, J.C. Meiske, W.R. Dayton, J. Anim. Sci. 74 (1996) 372–379.
[21] H. Kirschke, A.J. Barrett, N.D. Rawlings, Protein Profile 2 (14) (1992) 1581–1643.
[22] M. Kleinova, P. Zoellner, H. Kahlbacher, W. Hochsteiner, W. Lindner, J. Agric. Food Chem. 50 (2002) 4769–4776.
[23] P. Kugler, G. Vornberger, Histochemistry 85 (2) (1986) 157–161.
[24] I.G. Lange, A. Daxenberger, H.H.D. Meyer, AMPIS 109 (2001) 53–65.
[25] S.J. Lee, A.C. McPherron, Curr. Opt. Genet. Dev. 9 (1999) 604–607.
[26] M.E. Lippman, in: J.D. Wilson, D.W. Foster (Eds.), Textbook of Endocrinology, 7th ed., 1985, pp. 1309–1326.
[27] K.J. Livak, T.D. Schmittgen, Method 25 (2001) 402–408.
[28] H.H.D. Meyer, Handbook of experimental pharmacology, in: M. Oettel, E. Schillinger (Eds.), Estrogens and Antiestrogens, vol. 135/II, Springer-Verlag, Berlin, Heidelberg, 1999.
[29] H.H.D. Meyer, APMIS 109 (2001) 1–8.
[30] C. Moran, J.F. Quirke, D.J. Prendiville, S. Bourke, J.F. Roche, J. Anim. Sci. 69 (1991) 4249–4258.
[31] O. Mueller, S. Lightfoot, A. Schroeder, Agilent Technologies, 2004. Application 5989-1165EN.
[32] K. Nakashima, T. Komatsu, M. Yamazaki, H. Abe, J. Nutr. Sci. Vitaminol. 51 (4) (2005) 248–253.
[33] S.A. Ness, Biochem. Biophys. Acta 12788 (1996) F123–F139.
[34] Y. Ono, K. Kakinuma, F. Torii, A. Irie, K. Nakagawa, S. Labeit, K. Abe, K. Suzuki, H. Sorimachi, J. Biol. Chem. 279 (4) (Jan 23 2005) 2761–2771.
[35] A. Pardo, M. Selman, Int. J. Biochem. Cell Biol. 37 (2005) 283–288.
[36] M.W. Pfaffl, A. Daxenberger, M. Hageleit, H.H.d. Meyer, J. Vet. Med. A 49 (2002) 57–64.
[37] M.W. Pfaffl, I.G. Lange, H.H.D. Meyer, J. Steroid Biochem. Mol. Biol. 84 (2003) 159–166.
[38] H. Penzlin, Lehrbuch der Tierphysiologie, 7, Auflage, Elsevier, Spektrum Akademischer Verlag, 2005.
[39] T. Pollard, W. Earnshaw, Cell Biology, Saunders, 2002.
[40] J. Pottier, C. Cousty, R.J. Heitzman, I.P. Reynolds, Xenobiotica 11 (1981) 489–500.
[41] D. Randall, W. Burggen, K. French, W.H. Eckert, Animal Physiology, Freeman and Company, 2002.
[42] P.J. Reeds, S.M. Hay, P.M. Dorward, R.M. Palmer, Br. J. Nutr. 56 (1986) 249.
[43] I. Richard, C. Roudaut, S. Marchand, S. Baghdiguian, M. Herasse, D. Stockholm, Y. Ono, L. Suel, N. Bourg, H. Sorimachi, G. Lefranc, M. Fardeau, A. Sebille, J.S. Beckmann, J. Cell Biol. 151 (7) (2000) 1583–1590.
[44] F.J. Rose, Environ. Qual. Saf. Suppl. 5 (1976) 227–237.
[45] H. Sorimachi, S. Ishiura, K. Suzuki, Biochem J. 328 (1997) 721–732.
[46] D.J. Tobin, K. Foitzki, T. Reinheckel, L. Mecklenburg, V.A. Botchkarev, C. Peters, R. Paus, Am. J. Pathol. 160 (5) (2002) 1807–1821.
[47] J. Vandekerckhove, G. Bugaisky, M. Buckingham, J. Biol. Chem. 261 (4) (Feb 5 1986) 1838–1843.
[48] J. Vandekerckhove, K. Weber, J. Mol. Biol. 126 (4) (Dec 25 1978) 783–802.
[49] D. Voet, J.G. Voet, C.W. Pratt, Lehrbuch der Biochemie, Wiley-VCH, 2002.
[50] P. Zhe, K. Hoguen, J. Bong Kyun, L. Woo Jung, P. Chanil, Cancer 80 (1997) 865–872.

Appendix II

EFFECTS OF PLATE POSITION, PLATE TYPE AND SEALING SYSTEMS ON REAL-TIME PCR RESULTS

M. Reiter, M. Pfaffl
Physiology Weihenstephan, TU München, Weihenstephaner Berg 3, D-85354 Freising
Correspondence to: Martina Reiter
E-mail:martina.reiter@wzw.tum.de

ABSTRACT

Real-time PCR has become an important tool for quantitative nucleic acids analysis. By now, a wide rang of real-time PCR instruments; PCR plastic consumables and sealing systems are available. In this study both transparent and white plates were taken to test possible position and plate effects on quantitative PCR results on different real-time platforms: ep realplex (Eppendorf, Hamburg, Germany) and the iQ5 (Bio-Rad, Palo Alto, USA). Heat and adhesive sealing was used to compare sealing systems. Heterogeneity in Ct values was calculated to show possible effects of plate type and sealing system. Heterogeneity in amplification efficiency should show possible positional effects of 96-well plates.
White plates showed higher amplification efficiency because of higher fluorescence reflection. This had no significant effect on the PCR efficiency but on the sensitivity of the quantification assay. Constant positional effects concerning wells, columns or rows could not be detected.

Keywords: real-time qRT-PCR, position effects, plate type, sealing system

Introduction

Polymerase chain reaction (PCR) has become an important tool in molecular diagnostics, so also the range of products for PCR analysis has increased (4). Nowadays various real-time PCR instruments from different providers are available. Two main instrument platforms exist, the plate (96-well, 384-well) or rotor systems (32-well, 36-well, 72-well, 100-well). Additionally the instruments have different light sources, detection systems, thermocycling mechanism and software (2).

Especially for the plate systems various plastics consumables are available. From tubes to strips with caps, 96-well plates, 384-well plates, transparent or white, with adhesive or heat sealing film, the customer has a wide range of possibilities. White PCR plates are often recommended by the manufacturers because of lower autofluorescence and to achieve better quantification results (5).

It is known that positional effects on plates can appear during a PCR run, which can occur between different wells, columns or rows (7). This can increase the variation of measured fluorescence and therefore significantly influence the resulting PCR quantification.

In this study the effects of plate type and the sealing system on qPCR results should be investigated. Additionally possible positional effects on 96-well plates should be regarded. Two types of 96-well plates (white and transparent) and different sealing systems (self adhesive and heat sealing) were analysed in different real-time PCR instruments: iQ5 (Bio-Rad, Hercules, U.S.A) and ep realplex (Eppendorf, Hamburg, Germany) 96-well platforms.

A first experiment was done to show possible position effects of a 96-well plate and to calculate heterogeneity of a qPCR run. A second experiment was performed to show the differences in qPCR efficiency between transparent and white plates. In both experiments different sealing systems were used to compare the differences between adhesive and heat sealing films.

Material and Methods

Experiment 1

Position and plate effects were analysed using transparent and white semi-skirted, 250µl, twin.tec PCR plate 96 (Eppendorf). Plates were sealed using adhesive PCR film and heat sealing film (Eppendorf). Real-time amplification and quantification was done in the iQ5 (Bio-Rad) and in the Mastercycler ep realplex (Eppendorf) systems. One biological sample was measured in 96 replicates to evaluate the intra-run variability (n=96), analysing one transcript. All measurements were repeated in three plate replicates to evaluate the inter-run differences (n=3).

Experiment 2

Influence of plate type was analysed in a second experiment, using transparent and white semi-skirted, 200µl, Thermo-Fast 96-well PCR plates (ABgene, Surrey, UK). Plates were cut in three-column peaces, wherefore both the transparent and the white plate parts could be measured in parallel in one PCR run (**Fig. 1**). Plates were sealed using adhesive PCR film (ABgene) and real-time amplification and quantification was done in the iQ5 real-time system (Bio-Rad). Each column included the standard curve (S1-S6:10^7-10^2 copies), the unknown biological sample (U) and a non template control (NTC, RNAse free water). On one plate type the measurement was so repeated

six times (n=6) within one run. The experiment was repeated three times (n=3).

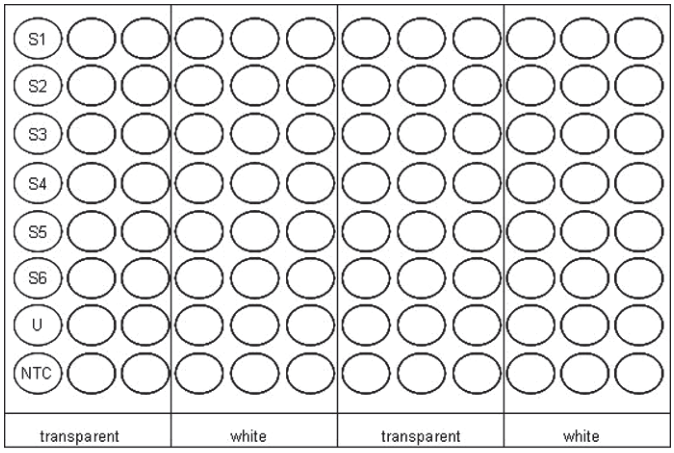


Fig. 1. Plate setup of experiment 2 for the measurement of transparent and white plates in one run. Adhesive PCR film was taken for sealing. Baseline and threshold adjustment was calculated for each column peace separately by the iQ5 optical system software, version 2.0 (Bio-Rad). S1-S6 contain the standard curve from 10^7 to 10^2 start copies, U represents the unknown biological sample and NTC the non-template control consisting of RNAse free water. Column setup was consistent over the entire plate.

Biological sample and standard curve

Total RNA was isolated from *m. triceps* of *macaca fascicularis* using TriFast (peqLab Biotechnologie GmbH, Erlangen, Germany). The standardized protocol from the supplier was used for the extraction. To quantify total RNA concentration, optical density (OD) was measured with the Nanodrop (peqLab). Total RNA integrity and quality control was performed with the Bioanalyzer 2100 (Agilent Technology, Palo Alto, USA) (3).

For the standard curve of experiment 2 the RT-PCR product of experiment 1 was collected. For DNA extraction and purification Wizard SU Gel and PCR Clean-Up System from Promega (Madison, USA) was used. DNA concentration was measured with the Nanodrop (PeqLab) and the standard curve down to 100 copies was generated.

Reverse transcription and quantitative PCR

Reverse transcription was done with 1µg total RNA using 5xRT buffer, dNTPs (10mM), random hexamer (50µM) primers and 200 U/µl M-MLV Reverse Transcriptase, RNase H minus (Promega, Madison, U.S.A). PCR was performed using MESA GREEN qPCR MasterMix Plus for SYBR Assay No ROX w/ fluorescein Kit (Eurogentec, Köln, Germany). Total PCR reaction volume was 15µl. In both experiments cathepsin L (CTSL) transcript was quantified. Pipetting was performed using the CAS-1200 robot and conductive pipette tips using liquid level sensing (LLS) technology (Corbett Life Science, Sydney, Australia).

Data analysis and statistics

In the iQ5 Ct-values and amplification data were analysed using the Bio-Rad iQ5 optical system software, version 2.0 (Bio-Rad). Ct values were determined with "PCR Base Line Subtracted Curve Fit" analysis method of the software, using fluorescein normalized data.

In the ep realplex instrument data were analysed using eppendorf realplex software, version 1.5 (Eppendorf). Ct-value was calculated using "Noiseband" algorithm of the instrument software.

Calculation of amplification efficiency in experiment 1

In the first experiment amplification efficiency was calculated as a value of the second derivative in its local discrete maximum after the amplification data was smoothed with a sigmoid fit (8) as follows.

$$f''(n) = \frac{a}{b^2} \cdot \frac{e^{\frac{n-n_0}{b}} - e^{-2\frac{n-n_0}{b}}}{\left(1+e^{\frac{n-n_0}{b}}\right)^3}$$

$$f = y_0 + \frac{a}{1+e^{-(\frac{n-n_0}{b})}}.$$

Where the a (plateau height), b (slope), n_0 (inflexion point of curve), are the obtained parameters of the fitted smoothing model. The natural logarithm base is described by e, n shows the actual fluorescence number and y_0 shows the ground fluorescence.

Calculation of PCR efficiency in experiment 2

In the second experiment PCR efficiency (E) of the qPCR reaction was calculated by the instruments software, on the basis of the slope of the standard curve and the equation:

$$\mathbf{E = 10^{-1/slope} - 1}$$

Results and Discussion

Heterogeneity in Ct values in experiment 1

In both instruments the transparent, adhesive sealed plates showed the highest Ct variations (**Table 1**). The iQ5 instrument showed lower inter-run standard deviations (S.D=0.28) within the different plate setups compared to the ep realplex (S.D.=0.40);(p-value=0.302). This may be the advantage of fluorescein normalized Ct data. For the iQ5 instrument the use of special fluorescein kits is recommended to decrease data variability. In other instruments ROX dye can be taken if problems with high data variations appear.

Heterogeneity in amplification efficiency in experiment 1

No pattern or significant homogenous position effects (wells, columns, rows) could be detected within the different runs and instruments on the 96-well plates in experiment 1 (**Fig. 2a, 2b**).

The amplification efficiency (AE) was significantly higher in the white plates than in the transparent plates (p-value=0.001), due to the better fluorescence reflection in the white plates. There were no significant differences between the amplification

TABLE 1

Intra-run and inter-run variations were calculated for the Ct values and the amplification efficiency (AE). Additionally standard deviations (S.D.) and coefficients of variation (C.V.) of all runs were determined for the iQ5 and the ep realplex for the transparent, heat sealed plates, (hsEPD), the white, heat sealed plates (hsEPW), the transparent, adhesive sealed plates (asEPD) and the white, adhesive sealed plates (asEPW).

iQ5												
	intra-run data			**intra-run data**			**intra-run data**			**inter-run data**		
Ct	Ct	S.D.	C.V. (%)	Ct	S.D.	C.V. (%)	Ct	S.D.	C.V. (%)	**Ct**	**S.D.**	**C.V. (%)**
AE	AE	S.D.	C.V. (%)	AE	S.D.	C.V. (%)	AE	S.D.	C.V. (%)	**AE**	**S.D.**	**C.V. (%)**
	hsEPD											
Ct	26.35	0.20	0.75	24.60	0.15	0.60	24.56	0.16	0.65	**25.17**	**0.17**	**0.67**
AE	75.50	9.88	13.09	86.60	6.46	7.45	73.90	5.74	7.77	**78.67**	**7.36**	**9.36**
	hsEPW											
Ct	26.38	0.16	0.62	24.50	0.18	0.74	24.45	0.32	1.31	**25.11**	**0.22**	**0.89**
AE	1213.60	82.02	6.76	1375.20	80.55	5.86	1411.60	139.61	9.89	**1333.47**	**100.73**	**7.55**
	asEPD											
Ct	25.23	0.19	0.76	25.25	0.79	3.12	25.12	0.69	2.74	**25.20**	**0.56**	**2.21**
AE	73.20	10.09	13.79	72.10	14.40	19.97	72.50	10.54	14.54	**72.60**	**11.68**	**16.08**
	asEPW											
Ct	24.71	0.14	0.55	25.17	0.19	0.74	25.15	0.17	0.67	**25.01**	**0.16**	**0.65**
AE	1261.70	79.62	6.31	1285.20	84.74	6.59	1246.20	98.89	7.94	**1264.37**	**87.75**	**6.94**
									EPW	**25.06**	**0.19**	**0.77**
									EPD	**25.18**	**0.36**	**1.44**
									hs	**25.14**	**0.19**	**0.78**
									as	**25.10**	**0.36**	**1.43**
realplex												
	hsEPD											
Ct	25.11	0.25	1.01	24.56	0.44	1.77	25.15	0.41	1.65	**24.94**	**0.37**	**1.48**
AE	122.30	25.00	20.44	120.30	22.33	18.56	133.10	27.01	20.29	**125.23**	**24.78**	**19.79**
	hsEPW											
Ct	24.10	0.48	1.98	23.95	0.38	1.57	22.49	0.17	0.76	**23.51**	**0.34**	**1.44**
AE	1911.30	354.10	18.53	2013.10	345.81	17.18	2410.42	162.89	6.76	**2111.61**	**287.60**	**13.62**
	asEPD											
Ct	23.36	0.52	2.23	23.71	0.70	2.96	23.83	0.33	1.40	**23.63**	**0.52**	**2.20**
AE	181.55	28.68	15.80	188.30	19.90	10.57	177.80	24.79	13.94	**182.55**	**24.46**	**13.40**
	asEPW											
Ct	22.55	0.49	2.17	22.60	0.34	1.50	22.99	0.23	1.00	**22.71**	**0.35**	**1.56**
AE	3003.3	211.679	7.05	2916.20	301.80	10.35	2897.60	218.88	7.55	**2939.03**	**244.12**	**8.31**
									EPW	**23.11**	**0.35**	**1.50**
									EPD	**24.29**	**0.44**	**1.84**
									hs	**24.23**	**0.35**	**1.46**
									as	**23.17**	**0.44**	**1.88**

efficiencies between the instruments, comparing transparent and white plates (p-value$_{transparent}$=0.113: p-value$_{white}$=0.098).

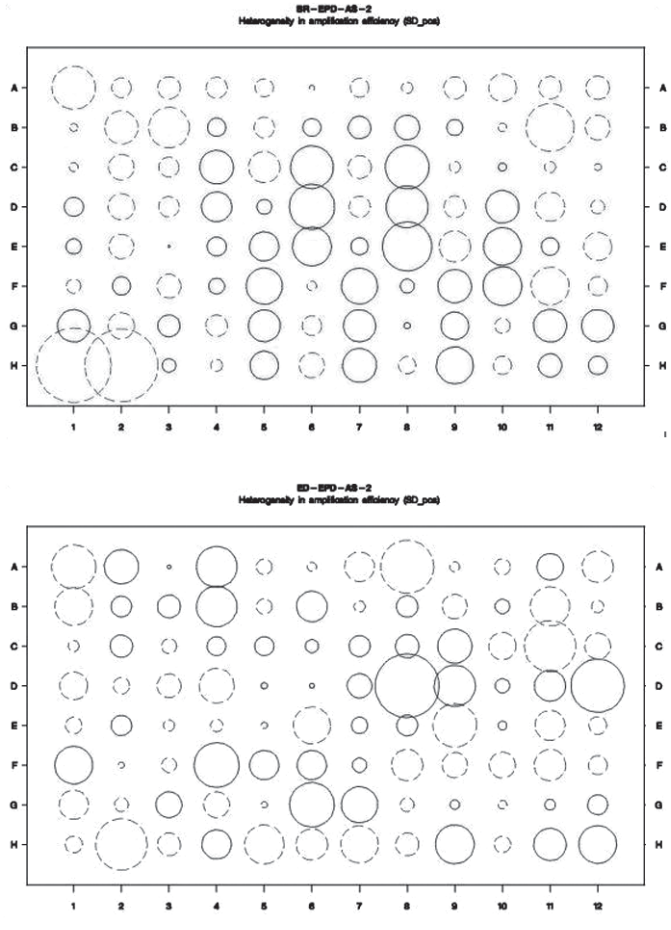


Fig.e 2a/2b. Amplification performance on basis of the Ct values determined with the iQ5 instrument (Figure 2a) and with the ep realplex (Figure 2b), using the transparent and adhesive sealed plate. Grey dashed circles show under-expressed, black solid circles over-expressed fluorescence levels from the plate mean expression. The size of the circles reflects the differences from the mean. No homogenous position effects (columns, rows) can be recognised.

The amplification efficiency (AE) was significantly higher in the white plates than in the transparent plates (p-value=0.001), due to the better fluorescence reflection in the white plates. There were no significant differences between the amplification efficiencies between the instruments, comparing transparent and white plates (p-value$_{transparent}$=0.113: p-value$_{white}$=0.098).

Heterogeneity in qPCR efficiency in experiment 2

The results of experiment 2 showed in average 5.2 fold higher fluorescence levels (relative fluorescence unit) in the white plates compared to transparent. To see if the higher fluorescence level also induces higher qPCR efficiency, the standard curves on white and transparent plates were regarded. No significant differences between PCR efficiencies (p-value=0.437, n=6) could be seen between transparent ($E_{transparent}$=97.5%) and white (E_{white}=96.0%) plates.

White plates showed higher slopes of the standard curve (slope$_{white}$=-3.441) than transparent plates (slope$_{transparent}$-3.387); (p-value=0.243). This effect induced lower PCR efficiencies (**Fig. 3**).

The y-intercept, representing the theoretical threshold cycle to detect one start molecule, trend to be significant between plate types (p-value=0.078). Y-intercept of the white plates was in average 1.109 cycles lower (y-intercept$_{white}$=33.707 cycles) than in the transparent plates (y-intercept$_{transparent}$=34.816 cycles). Implementing the calculated mean PCR efficiencies of the different plate types and the 1.109 cycles difference, an increase in sensitivity by 2.75 fold (n-fold=$1.975^{34.861}/1.960^{33.707}$) could be indicated.

Therefore the higher autofluorescence of white plates has benefical effects on qPCR sensitivity but not directly on qPCR efficiency.

Handling

Experiment 2 with adhesive sealing film showed time by time evaporated samples within one run mainly at the boarder positions. The fixing of the sealing film at the plate boarders with the use of a sealing rubber (ABgene, Surrey, UK) could not avoid sample evaporation. Only the use of a special optical compression pad (Bio-Rad) could keep the adhesive sealing film on the plate.

To avoid evaporation of the samples and contamination of the instrument heat sealing systems should be preferred. With

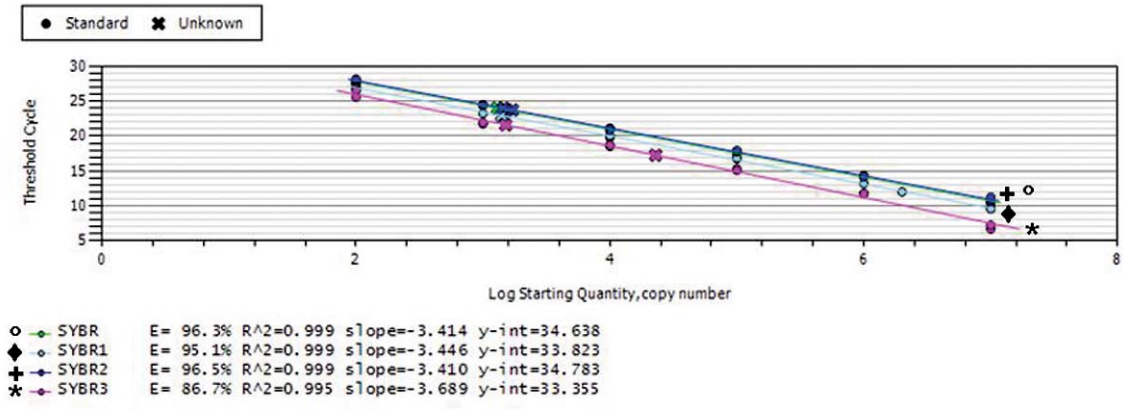


Fig. 3. Standard curves of experiment 2. SYBR (°) shows the mean efficiency calculation for column 1-3 (transparent), SYBR 1 (♦) for column 4-6 (white), SYBR2 (+) for column 7-9 (transparent) and SYBR 3 (*) for column 10-12 (white), performed on the Bio-Rad iQ5 real-time cycler.

the heat sealed plates in experiment 1, evaporation of samples was not recognized in one of the samples.

Conclusion

Validity of the final experimental mRNA expression results is dependent on the variability of the measurement system. Total data variation appears in every experiment and consists from error accumulation of the different experimental steps, starting at sampling, storage, nucleic acid extraction, reverse transcription, and quantitative qPCR. Possibilities to reduce error accumulation in reverse transcription and qPCR were already shown by Ståhlberg et al. (6).

In this study it was shown that also the selection of PCR plastics consumables and sealing systems can influence data variability and sensitivity of the quantification.

White plates showed higher amplification efficiency because of higher fluorescence reflection. This had no significant effect on the efficiency but on the sensitivity of the quantification assay. White plates are helpful in detection and quantification of low copy numbers in biological samples or when problems with the PCR dye concentration occur.

The sealing system seems to influence the variability of the quantification results. Adhesive PCR film sealing allows evaporation during PCR cycling procedure, resulting in high variations of Ct-values.

Regarding possible plate position effects, only single wells showed over or under estimated values from mean amplification efficiency but the expected border effects could not be shown statistically. In the ep realplex the 96 single LED systems seem to avoid these random effects, in the iQ5 the collection of well to well differences in the beginning of the run seems to be the crucial software tool.

REFERENCES

1. **Bio-Rad tech note 5266.** Improving reliability and reproducibility of real-time PCR reactions sealed with clear films. Bio-Rad Laboratories, 2005, Bulletin 5266.
2. **Dorak M.T.** (2006) Real-time PCR. Bios Advanced Methods, Taylor and Francis Group, N.Y., USA.
3. **Fleige S., Pfaffl M.W.** (2006). Mol A Med 2006, 27: 126-139.
4. **Kaltenboeck B., Wang C.** (2005) Adv Clin Chem, 40:219-59.
5. 4titude (2008). FRAMEstar®, 96 and 384 well PCR plates. Available via DIALOG www.4ti.co.uk/new%20site/images/4titude%20-%20Framestar.pdf
6. **Ståhlberg A., Håkansson J., Xiang X., Semb H., Kubista M** (2004) Clin Chem, 50(3):509-15.
7. **Sundberg R., Castensson A., Jazin E** (2006) Biostat, 7(1):130-144.
8. **Tichopad A., Didier A., Pfaffl M.W.** (2004) Molecular and Cellular Probes 18, 45-50.

Appendix III

First steps towards a new screening method for anabolic androgenic agents in human hair follicle

M. Reiter[1], S. Lüderwald[2], M.W. Pfaffl[1], H.H.D. Meyer[1]

[1]Physiology Weihenstephan
Technische Universität München
Weihenstephaner Berg 3
85354 Freising
Germany

[2]Institute of forensic medicine
Universität München
Nussbaumstr. 26
80336 München

Corresponding author:

Martina Reiter
Physiology Weihenstephan
TU München
Weihenstephaner Berg 3
85354 Freising
martina.reiter@wzw.tum.de
Tel.:+498161713867
Fax:+498161714204

Abstract

Never before doping has been such a topic as it is today and the interests in more stringent and periodic doping controls is very high. The existing doping tests are restricted to methods based on mass spectrometry, analysing the drug in different tissues like urine, blood or hair. The idea of this feasibility study in hair follicle was to test a possible new way of detecting anabolic steroids, based on biomarker measurement for different anabolic agents. It is known that anabolic steroids can influence mRNA expression of certain genes that results in androgen specific effects. With specific biomarker expression patterns it should be possible to foster the existing analysis methods. The hair follicle was chosen because it is known to be influenced by anabolic steroids, and would be easy to be taken for doping controls.

The first aims were to develop a method which makes it possible to analyse gene expressions in plucked hair follicle samples via quantitative real-time RT-PCR (qRT-PCR). Additionally it should be analyzed which cell types of the hair follicle are taken by the plucking procedure. In a first study samples from untreated women and men were taken to find gender specific differences and in a second study also samples from weight lifters under doping conditions could be taken and were compared with a control group. As biomarkers, different androgen dependent target genes expressed in the hair follicle were analyzed: The steroid receptor group including the androgen receptor (AR), estrogen receptor (ERα) and glucocorticoid receptor (GR). Additionally the hair cycle factors, the fibroblast growth factors FGF7 and FGF2 and interleukin1 beta, (IL1β) were analyzed. FasLigand receptor (FasR), Bcl-2, Caspases 3 and 8 were measured as apoptosis group. 5α- steroidreductase (SRD5A2) represented the enzyme group. For a relative quantitation three reference genes beta-actin (ACTB), glycerolaldehyde-3-phsophate-dehydrogenase (GAPDH) and ubiquitin (UBQ) were taken. To show which part of the hair follicle was taken by plucking, the hair follicles were haemalaun-eosin stained.

The hämalaun-eosin stain clearly showed that hair follicle cells from the root sheath could be taken by plucking but without taking the hair bulb that includes the hair papilla. In the first study no significant gender dependent differences in the regulation of the measured target genes could be detected. In the second study a down-regulation of the GR could be calculated in the treated hair samples and FGF7 was only expressed in treated samples.

These results show first promising differences between treated and untreated samples, a precondition for the development of a possible screening method for anabolic agents. The ambitions for the future should be the identification of specific biomarker patterns for functional groups of anabolic agents, in order that abuses can be uncovered.

Key words: anabolic androgenic agents, hair follicle cells, quantitative real-time RT-PCR

Introduction

The various doping scandals of the cyclist, especially during the “Tour de France 2007” clearly showed that doping is a big topic in competitive sport and doping controls need to be more universal in detecting drug misuse. Beside doping methods that increase the oxygen transport capacity in blood, especially the use of anabolic androgenic agents (AAS) is very popular. These substances are supposed to increase muscle mass via nitrogen retention resulting in a better performance (Hartgens et al., 2004; Kuhn 2002). The International Olympic Committee (IOC) has already banned the use of AAS by athletes in 1974 and methods for detecting and identifying these substances are developed continuously. Most of them are mass-spectrometry based, but there are still problems with the doping control procedure. Tissues like urine, blood and hair are taken for these analyses. They are easy to be collected and include the drugs or its metabolites. Taking the existing analysis methods the question how to screen for unknown designer drugs and its metabolites remains open. It would be helpful to have additional methods which broaden the existing screening spectrum (Catlin et al., 1997; Schänzer 1996). The effects of xenobiotic steroids in the organism are well documented and it is known that these testosterone analoga can act via mRNA expression in the cell, influencing the expression of different genes (Reiter et al., 2006). In the hair follicle androgenic hormones act via the dermal papilla but also initiate different expression pathways in the cells of the inner and outer root sheath (Stenn and Paus 2001). The idea of this feasibility study was to find a way to extract RNA from cells of hair follicle samples obtained by plucking the hair of the androgen dependent frontal scalp. The identification of different target genes could then be a possibility to identify a biomarker pattern. Taking this androgen specific pattern the intake of illegal drugs like testosterone and other xenobiotic steroids could be possible.

To make sure that cells of the hair follicle can be extracted by plucking the hair, histological slices were gained and stained to identify the cell types of the hair follicle.

Possible biomarkers (target genes) which are known to be influenced by anabolic agents and which are expressed in the dermal papilla or hair follicle were taken and separated in functional groups: Different studies have mentioned the important role of estrogens in the hair cycle, especially the estrogen receptor alpha (ERα) that mediates catagen induction of 17β-estradiol in the hair follicle. It could be detected not only in the dermal papilla cells but also in matrix keratinocytes and inner and outer root sheath (Chanda et al., 2000; Ohnemus et al., 2005). Two other important receptors of the AAS signaling pathways in the dermal papillae are the androgen receptor (AR) and the glucocorticoid receptor (GR). Through the binding to the receptors the androgens induce the mRNA expression of different genes (Lachgar et al.; 1999; Karstila et al., 1994).

Factors that inhibit hair follicle growth are the fibroblast growth factor 2 (FGF2) which is expressed in the hair follicle and interleukin 1β (IL1β) that is located in the epidermis and cells of the root sheath. Fibroblast growth factor 7 (FGF7) is the antagonist and induces hair follicle growth (Mitusi et al., 1997; Ota et al., 2002). Also apoptosis factors play a crucial role in the catagen phase of the hair growth. Bcl-2 was found to be continuously expressed in the hair papilla and plays a role as a support and signal center, and apoptosis related receptors like FasR were analyzed in the follicle epithelium. Also other factors of the extrinsic apoptosis pathway Caspase 3 and 8 were tested (Stenn and Paus 2001).

5α-reductase (SRD5A) is the enzyme that catalyses the transformation of testosterone to 5α-dihydrotestosterone, an androgen that is supposed to be a mediator of hair loss. Two isoenzymes type 1 and type 2 exist whereas type 1 is mainly expressed in dermal papilla cells, type 2 also in dermal fibroblast. Additionally 5α-reductase type 2 is restricted to beard and frontal scalp (Itami and Inui 2005; Lachgar et al., 1999; Skalba et al., 2006).

Material and Methods

Hair follicle extraction

Frontal scalp hair follicle samples were taken from the androgen dependent upper part of the head. In the first study five hair follicle samples were taken from women and five samples from men to find possible gender specific differences. In the second study three samples from weight lifters that are known to take different anabolic agents (testosterone, trenbolone) could be taken and three hair samples from untreated man served as control.

To get the hair follicle, five to six hairs were penned in a clamp, plucked and cut to ca. 1 cm length. The hairs were immediately given in a tube filled with lysis buffer (MasterPure RNA Purification Kit, Epicentre Biotechnologies, Madison, WI, U.S.A.) and shock frozen in liquid nitrogen. After freezing the samples were stored at -80°C. To extract the RNA from the hair samples, the MasterPure RNA Purification Kit and protocol (Epicentre Biotechnologies, Madison, WI, U.S.A.) was used.

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

RNA quality

To get an idea about the RNA quality of the extracted total RNA, RNA integrity and quality control was performed with the Bioanalyzer 2100 (Agilent Technology, Palo Alto, USA). Samples from both studies were analyzed and taken as reference for an average RNA quality. For sample analysis eukaryotic total RNA Nano Assay (Agilent Technology) was taken and the RNA Integrity Number (RIN) served as RNA quality parameter (Fleige et al., 2006).

Primer design and testing

All primers were designed using published nucleic acid sequences of Ensembl Genom Browser (http://www.ensembl.org) and NCBI (www.ncbi.nlm.nih.gov). Primer 3 (http://frodo.wi.mit.edu/) was taken to design and optimize all prime pairs with regard to primer dimer formation, self-priming formation and primer annealing temperature at 60°C (table 1). Designed primers were ordered and synthesized at MWG Biotech (Ebersberg, Germany). All selected target genes were first established in a cell culture study for HFDPC (hair follicle dermal papilla cell) samples (Reiter et al., manuscript submitted).

Real-time qRT-PCR

Quantitative real-time RT-PCR was performed in the Rotor Gene 6000 (Corbett Life Science, Sydney, Australia) using SuperScript III Platinum SYBR Green One-Step qPCR Kit (Invitrogen, Carlsbad, USA) by a standard protocol. Samples were diluted to 10ng/µl and 50ng tRNA in 5µl (Invitrogen, Carlsbad, USA) was added to each sample to protect mRNA of degradation. 38 ng total RNA were taken for one PCR reaction in a total volume of 10µl. Crossing points (Ct) and melting curves were acquired by using the *"quantitation"* and *"melting curve"* program of the Rotor-Gene 6000 analysis software.

Only genes with clear and single melting peaks were taken for further data analysis. Samples with irregular melting peaks were excluded from the calculation. All samples were baseline corrected and threshold was set manually, using same threshold levels for one gene in all samples.

Histological stain and immunohistochemistry

Hair samples were incorporated in frozen section medium (Richard-Allan Scientific, Kalamazoo, USA) and prepared using a cryostat mycrotom HM 505E (Micron, Walldorf, Germany). 6 µm slices of the hair follicles were fixed on the object holder with 100%, -30°C frozen ethanol and stained in haematoxylin (Sigma, Germany) for 3 min. The histological slices were blued with tap water, stained in 1% eosin (Sigma-Aldrich, Munich, Germany) for 7 min, washed with 50-100% ethanol and finally given in rotihistol (Sigma-Aldrich, Munich, Germany). With EUKITT the cover slips were fixed on the object holder and hardened over night.

Data Analysis and Statistics

Data were processed applying relative quantification method comparable to the ΔΔCt-method ($2^{\Delta\Delta Ct}$) (Livak et al, 2001). For normalization of target gene (TG) expression the arithmetic mean of the following non regulated reference genes (RG) were taken for the calculation of a reference gene index (RGI): In study one (RG1) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and (RG2) ubiquitin (UBQ), in study two (RG1) ACTB and (RG2) UBQ. For every sample gene expression of the two RG were analyzed and the mean value served as reference gene index.

In Excel (Microsoft, USA) all calculations were done. T-test calculations were used to find gender specific regulations of the target genes. In study one only ΔCt could be calculated because no control group existed. In study two ΔΔCt and the expression ratio could be analyzed.

$\Delta Ct = Ct_{(TG)} - Ct_{(RGI)}$

$\Delta\Delta Ct = \Delta Ct - \Delta Ct_{(Control)}$

$Ration = 2^{-\Delta\Delta Ct}$

Results

Histological stain

Under the microscope the slices of the plucked hairs showed a clear hair shaft with accumulated cells that are supposed to be epithelial cells from the root sheath, especially the outer root sheath. The whole hair bulb is missing what was expected because by plucking this part remains to build up a new hair (Figure 1).

RNA concentration and quality of hair follicle samples

Mean total RNA concentration of all samples of the first study was 674.6±520 ng, 741.4±546.6 ng in male and 676.8±509.7 ng in female samples. In the second study mean RNA concentration was 1144±64.4 ng.

The measured RIN values showed and average value of 7.9 what is high enough to expect good results in qRT-PCR.

PCR analysis of hair follicle samples

In the first study the reference genes GAPDH and UBQ and in the second study the reference genes ACTB and UBQ were not significantly regulated and could be taken for statistical calculation.

In the first study the target genes AR, ERα, FGF2, FGF7, IL1β, SRD5A2, FasR, Bcl-2, Caspase 8 and 9 were measured, in the second study AR, ERα, FGF7, FasR, Caspase 3 and 8. AR, ERα, FGF2, SRD5A2. The melt curve analysis of AR, ERα, SRD5A2 and Caspase 8 did not show a specific product. FGF7 only showed a specific product in the treated samples of study two, in the control samples the melt curve analysis showed no specific peak (Figure2).

Data analysis and statistics

In study 1 none of the measured target genes showed significant differences ($p\text{-value} < 0.05$) between the male and female samples in the first study. In the second study a significant down-regulation of 41 % for the GR could be calculated (p-value=0.02). For FGF7 the treated samples had a mean Ct of 23.9 cycles and a mean ΔCt-value of 4.88. This shows a lower expression compared to the reference genes.

Discussion

Different studies have shown that anabolic agents influence hair growth through the dermal papilla via regulation of different genes in males and females. The gene expression regulations induced in the papilla also showed effects on growth factors of root sheath cells (Obana et al., 2007; Randall et al., 1992; Robia et al., 2003). Analyzing these specific regulations in hair follicle samples could be a new possibility to identify the intake of forbidden anabolic agents like testosterone and other xenobiotic.

Histological stains of plucked hair follicle samples clearly showed that cells of the root sheath, especially the outer root sheath could be gained and used for RNA extraction without taking a skin biopsy. This is a pre-condition for the use in doping analysis because sampling has to be simple practicable. Samples from the androgen dependent part of the scalp (frontal scalp hair) were taken to show possible gender specific differences in gene expression. It was not possible to gain the hair papilla by plucking as expected. This could explain that AR, ERα and SRD5A2, target genes that are known to be mainly expressed in the hair papilla could not be detected.

To get hair follicle samples from weight lifters that own up to take anabolic agents is quite difficult; therefore only three samples could be analyzed and taken for statistical analysis. However, first significant differences between the treated and untreated samples could be detected. A first androgen dependent effect could be seen in the expression of FGF7 that could only be measured in the samples of the treated weight lifters, all untreated samples from study one and two did not show a specific product in qRT-PCR analysis.

It is known that androgens can bind and act via the GR what can be confirmed by the significant down-regulation of the receptor in the second study.

The expression of IL1β and Bcl-2 in the first study and FasR in both studies in the inner and outer root sheath of the hair follicle could be approved. (Karstila et al., 1994; Sohn et al.; 2007, Stenn and Paus 2001).

Summarizing, the development of a sampling and RNA extraction method for hair follicle samples and the measurement of first target genes, could be successfully accomplished.

Conclusion

With this study a first step towards a possible new screening method for anabolic androgenic agents via gene expression analysis could be done, with the aim to support existing, mass spectrometry based detection methods. Plucking hairs and taking the RNA from the hair follicle seems to be a practicable method to analyze different gene expression and find specific biomarkers. A sampling and extraction method could be successfully developed and first target genes could be analyzed. Differences in gene expression regulations between the different genders could not be calculated. It seems that only very high androgen concentrations significantly influence these gene regulations. This effect could be seen in the expression of FGF7 and the significant regulation of GR only in samples of androgen treated athletes, what makes these two target genes to first biomarkers of AAS.

These results show first promising differences between treated and untreated samples, a precondition for the development of a possible screening method for anabolic agents. The ambitions for the future should be the identification of specific biomarker patterns for functional groups of anabolic agents, in order that abuses can be uncovered.

References

Catlin DH, Hatton CK, Starcevic SH. Issues in detecting abuse of xenobiotic anabolic steroids and testosterone by analysis of athletes´urine. Clinical Chemistry, 1997, 43:7.

Chanda S, Robinette CL, Couse JF, Smart RC. 17beta-estradiol and ICI-182780 regulate the hair follicle cycle in mice through an estrogen receptor-alpha pathway. Am J Physiol Endocrinol Metab, 2000, 278(2):E202-10.

Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Mol A Med 2006, 27: 126-139.

Hartgens F, Kuipers H. Effects of androgenic-anabolic steroids in athletes. Sports Med, 2004, 34(8):513-54.

Itami S, Inui S. Role of androgen in mesenchymal epithelial interactions in human hair follicle. J Invest Dermatol Symp Proc, 2005, 10(3):209-11.

Karstila T, Rechardt L, Honkaniemi J, Gustafsson JA, Wikströms AC, Karpinnen A, Pelton-Huikko M. Immunohistocytochemical localization of glucocorticoid receptor in rat skin. Histochemistry, 1994, 102(4):305-9.

Kuhn CM. Anabolic Steroids. Recent Prog Horm Res, 2002, 57:411-34.

Lachgar S, Charveron M, Sarraute J, Mourard M, Gall Y, Bonafe JL. In vitro main pathways of steroid action in cultured hair follicle cells: vascular approach. J Invest Dermatol Symp Proc, 1999, 4(3):290-5.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001, 25:402- 408.

Mitusi S, Ohuchi A, Hotta M, Tsuboi R, Ogawa H. Genes for a range of growth factgors and cyclin-dependent kinase inhibitors are expressed by isolated human hair follicles. Br J Dermatol, 1997, 137: 693-698.

Obana N, Chang C, Uno H. Inhibition of hair growth by testosterone in the presence of dermal papilla cells from the frontal bald scalp of the postpubertal stumptailed macaque. Endocrinology, 197, 138:356-361.

Ohnemus U, Uenalan M, Conrad F, Handjiski B, Mecklenburg L, Nakamura M, Inzunza J, Gustafsson JA, Paus R. Hair cycle control by estrogens: catagen induction via estrogen receptor (ER)-α is checked by ERβ signalling. Endocrinology, 2005, 146:1214-1225.

Ota Y, Sioth Y, Suzuki S, Ozawa K, Kawano M, Imamura T. Fibroblast growth factor 5 inhibits hair growth by blocking dermal papilla cell activation. Biochem Biophys Res Commun, 2002, 11;290(1):169-76.

Randall VA, Thornton MJ, Hamada K, Messenger AG. Mechanism of androgen action in cultured dermal papilla cells derived from human hair follicles with varying responses to androgens in vivo. J Invest Dermatol, 1992, 98:86S-91S.

Reiter M, Walf VM, Christians A, Pfaffl MW, Meyer HHD. Modification of mRNA expression after treatment with anabolic agents and its usefulness for gene expression-biomarkers. Anal Chim Acta, 2006, 586(1-2):73-81.

Robia CB, Mitteregger G, Aichinger A, Benesch M, Bamberg E. Effect of testosterone and oestradiol-17β on canine hair follicle culture. J Vet Med, 2003, 50:225-229.

Schänzer W. Metabolism of anabolic androgenic steroids. Clinical Chemistry, 1996, 42:7.

Skalba P, Dabkowska-Huc A, Kazimierczak W, Samojedny A, Samojedny MP, Chelmicki Z. Content of 5-alpha-reductase (type1 and type2) mRNA in dermal papillae from the lower abdominal region in women with hirsutism. Clin Exper Dermatol, 2005, 31:564-570.

Sohn KC, Jang S, Choi DK, Lee YS, Yoon TJ, Jeon EK, Kim KH, Seo YJ, Park JK, Kim CD. Effect of thioredoxin reductase 1 on glucocorticoid receptor activity in human outer root sheath cells. Biochem Biophys Res Commun, 2007, 11;356(11):810-5.

Stenn KS, Paus R. Controls of hair follicle cycling. Pharm Rev, 2001, 81 (1).

Appendix

Table 1: List of successfully analyzed target genes and reference genes in the hair follicle samples. Annealing temperature (AT) and the accession number in NCBI (Acces.Nr.) are listed.

Figure 1: Slice of the plucked hair with the hair shaft (1) and outer root sheath cells (2) which are clearly outlined by perifollicular sheath cells (3), stained in blue.

Figure 2: Melt curve of FGF7 in study 2 with the melt temperature on the x-axis and the first derivative of the melt curve on the y-axis. Control samples (green) do not show a specific product, treated samples (orange) show a specific melt peak at 83°C.

Table 1

Identity		**Sequenz 5´-3´**	**Amplicon (nt)**	**AT (°C)**	**Acces. Nr.**
IL1β	FOR	GGA CAG GAT ATG GAG CAA CAA G	121	60	NM000576
	REV	AAC ACG CAG GAC AGG TAC AG			
FasR	FOR	TTC TGC CAT AAG CCC TGT CC	174	60	NM000043
	REV	CCA CTT CTA AGC CAT GTC CTT C			
Bcl-2	FOR	GAG GAT TGT GGC CTT CTT TGA G	170	60	NM000633
	REV	ACA GTT CCA CAA AGG CAT CCC			
GR	FOR	TTC TGC GTC TTC ACC CTC AC	159	60	AH00275
	REV	CTG TCT CTC CCA TAT ACA GTC C			
FGF7	FOR	CCT GAG CGA CAC ACA AGA AG	167	60	M60828
	REV	GCC ACT GTC GCT TCC TTA TT			
UBQ	FOR	TGA AGA CTC TGA CTG GTA AGA CC	128	60	NM021009
	REV	CAT CCA GCA AAG ATC AGC CTC			
GAPDH	FOR	GAA GGT GAA GGT CGG AGT CAA	233	60	NM002046
	REV	GCT CCT GGA AGA TGG TGA TG			
ACTB	FOR	AGTCCTGTGGCATCCACGAAAC	78	60	NM01101
	REV	GCAGTGATCTCCTTCTGCATCC			

Figure 1

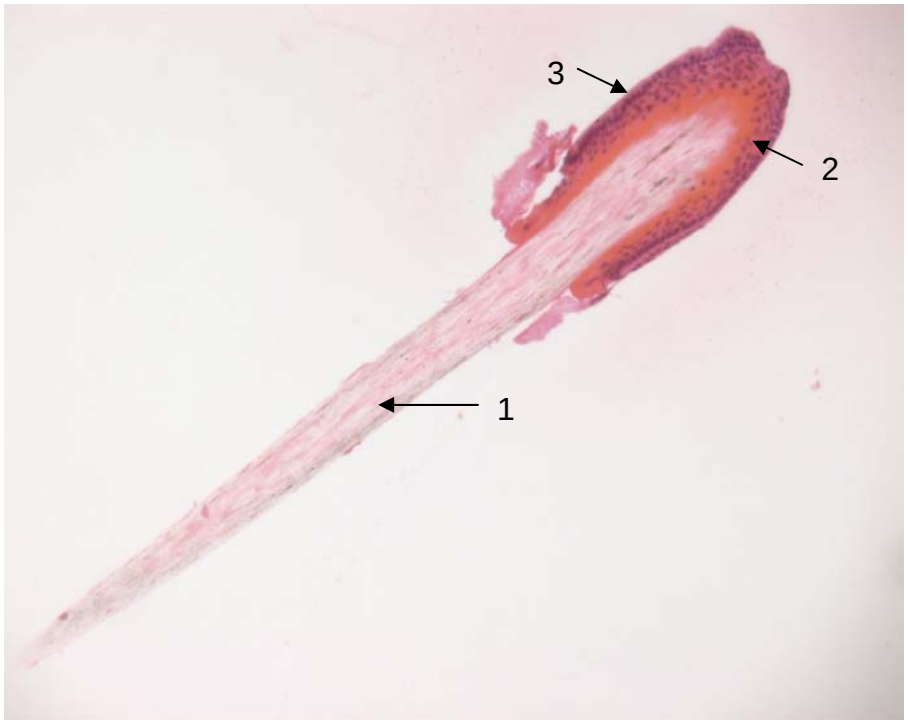
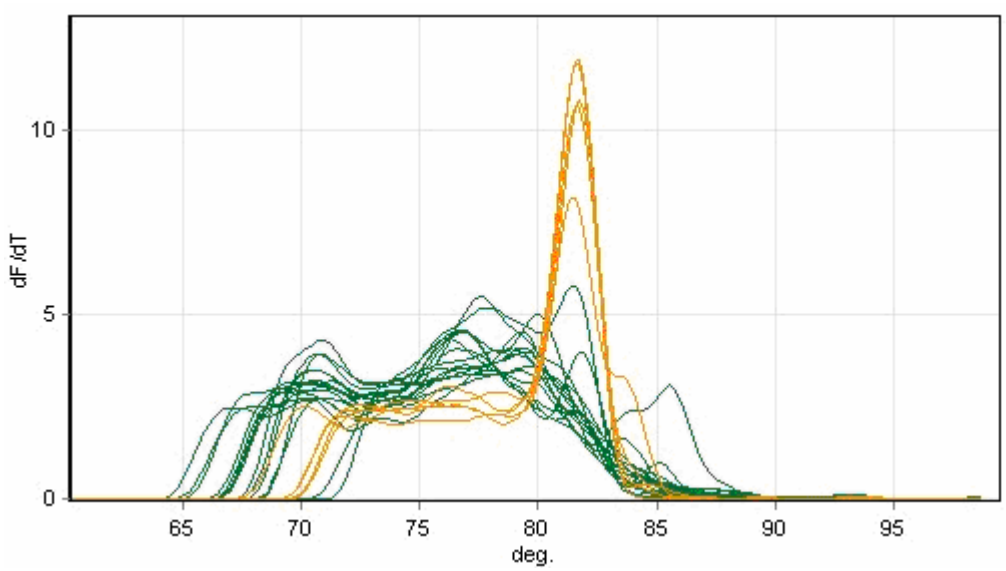


Figure 2



Appendix IV

Gene expression in hair follicle dermal papilla cells after treatment with stanozolol.

M. Reiter[1], M.W. Pfaffl[1], M. Schönfelder[2], H.H.D. Meyer[1]

[1]Physiology Weihenstephan
Technische Universität München
Weihenstephaner Berg 3
D-85354 Freising
Germany

[2]Institute of Public Health Research
Technische Universität München
Conollystraße 32
D-80809 Munich
Germany

Corresponding author:

M. Reiter
Physiology Weihenstephan
TU München
Weihenstephaner Berg 3
D-85354 Freising
martina.reiter@wzw.tum.de

Abstract

Doping with anabolic agents is a topic in sports where strength is crucial, e.g. sprinting, weight lifting and many more. Testosterone and its functional analogs are the drugs of choice taken as pills, creams, tape or injections to increase muscle mass and body performance, and to reduce body fat. Stanozolol (17β-hydroxy-17α-methyl-5α-androst-2-eno[3,2c]pyrazol) is a testosterone analogue with the same anabolic effect like testosterone but its ring structure makes it possible to take it orally. Therefore, stanozolol is one of the most frequently used anabolic steroids.

Common verification methods for anabolic drugs exist, identifying the chemicals in tissues, like hair or blood samples. The idea of this feasibility study was to search for specific gene expression regulations induced by stanozolol to identify the possible influence of the synthetically hormone on different metabolic pathways. Finding biomarkers for anabolic drugs could be supportive of the existing methods and an additional proof for illegal drug abuse.

In two separate cell cultures, human HFDPC (hair follicle dermal papilla cells) from a female and a male donor were treated with stanozolol. In the female cell culture treatment concentrations of 0nM (control), 1nM, 10nM and 100nM were chosen. Cells were taken 0h, 6h, 24h and 48h after stimulation and totalRNA was extracted. Learning from the results of the pilot experiment, the male cell culture was treated in 10nM and 100nM concentrations and taken after 0h, 6h, 24h and 72h. Using quantitative real-time RT-PCR expression of characteristics of different target genes were analysed.

Totally 13 genes were selected according to their functionality by screening the actual literature and composed to functional groups: factors of **apoptosis regulation** were Fas Ligand (FasL), its receptor (FasR), Caspase 8 and Bcl-2. Androgen receptor (AR) and estrogen receptor (ERα, ERβ) were summarized in the **steroid receptor group**. The **growth factor group** included the insulin like growth factor receptor (IGF1R) and growth hormone factor (GHR). Fibroblast growth factor 2 (FGF2) and keratinocyte growth factor (FGF7) were summarized in the **hair cycle factor group**. 5α-Steroidreductases (SRD5A1, SRD5A2) represented the **enzyme group**. Three **reference genes** were taken for relative quantification: ubiquitin (UBQ), glycerinaldehyde-3-phsophate-dehydrogenase (GAPDH), β-actin (ACTB).

In cell culture 1 AR, FasR, FGF2 showed significant regulations within one treatment time, significant gene expressions over time were analysed for Caspase 8. In cell culture 2 AR, FasR and SRD5A2 were significantly regulated within one treatment time.

In this feasibility study first biomarker for a screening pattern of anabolic agents could be identified providing the rationality to investigate modified, metabolic pathways in the whole hair follicle.

Keywords: anabolic agents**,** hair follicle dermal papilla cells, gene expression, qRT-PCR, mRNA

Introduction

Anabolic androgenic steroids (AAS) are misused by athletes because of their anabolic properties. Main functions of AAS are stimulation of protein synthesis and an antiglucorticoid effect which increases muscle mass and strength. They influence the central nervous system and increase motivation and performance (Duntas et al., 2003; Hartgens et al., 2004; Hickson et al., 1990; Griggs et al., 1989; Kuhn 2002). The International Olympic Committee banned the use of synthetic AAS in 1974 by athletes but today doping is still a big topic in almost all kinds of sport. The world anti doping agency (WADA) encourages drug testing laboratories to develop methods to detect AAS like testosterone, 17β-nortestosterone and stanozolol that are the most frequently found steroids in doping samples (Kuhn 2002; Clasing and Mueller 2006; Schänzer 1996). Common used methods in doping analysis are mass spectrometry (MS) based techniques, that directly detect the AAS or their metabolites in different tissues like urine, blood or hair samples. Especially the hair is a very interesting tissue for drug residue analysis because hormones are detectable over a long time and the samples are easily to collect by a non invasive manner (Gleixner et al., 1996; Catlin et al., 1997; Thieme et al., 2000; Ayotte 2006; Anielskie et al., 2005; Gambelunghe et al., 2007).
The intake of AAS influences the organism in many different ways. It can be supposed that these hormonally provoked changes in the metabolism can be seen on the level of mRNA gene expression (Reiter et al., 2007). Taking the hair root to analyse these expressions it is known that androgens act via the dermal papilla cells and influence different kind of growth factors and enzymes (Stenn and Paus 2001).
The aim of this feasibility study was to investigate regulated target genes in hair follicle cells to identify possible mRNA gene expression regulations aroused by stanozolol. Target genes were selected in functional groups to facilitate the identification of possible biomarker for the different hormones.

Materials and Methods

Reagent

The anabolic steroid stanozolol (S, 17β-Hydroxy-17α-methyl-androstano[3,2-c]pyrazole), was received from Sigma-Aldrich (Taufkirchen, Germany) with cell culture grade purity. For treatment the reagents was dissolved in 100% ethanol to a concentration 1mg/ml and diluted with cell culture medium to 1nM, 10nM and 100nM.

Experiment

Two HFDPC (hair follicle dermal papilla cell) cultures, one from a female (cell culture 1) and one from a male (cell culture 2) donor were treated in different concentrations with the anabolic steroid stanozolol and taken at different treatment time points.

Cell culture 1

Human HFDPC were ordered from Cell Applications (San Diego, USA). Cells had been cultivated from skin samples of the temple taken during plastic surgery at a 49 year-old, female patient. After establishing a primary cell culture the cells were frozen in serum-free freezing medium and sent cryopreserved (500,000 cells in 1ml). A ready-to-use HFDPC Medium and Supplement Kit (Cell Applications, San Diego, USA) was taken to cultivate the cells, containing Basal Medium, FCS (fetal calf serum), growth factors and antibiotics. For sub cultivation served a kit containing HBSS (HEPES buffered saline solution), trypsin/EDTA solution and neutralizing solution (Cell Applications, San Diego, USA).
The thawed cells were cultivated in collagen coated T-75 flasks (Cell Applications, San Diego, USA) containing 15ml HFDPC medium at 37°C in a humidified atmosphere of 5% CO_2 and sub-cultivated at 85% confluence. Cells were frozen and stored at -80°C after each splitting that was up to the sixth passage. For the experiment cells from third passage were thawed, given into 12-well plates (ca. $3.4x10^4$/well) and cultivated till 85% confluence. Cells were cultured in triplicates with 0nM (control-treatment), 1nM, 10nM and 100nM stanozolol groups on each plate, sampling took place at 0h (control-time), 6h, 24h and 48h.
To remove natural containing steroids for all experiments the FCS was stripped by using charcoal, as described by Darbre et al. (1983).

Cell culture 2

Human HFDPC cells were ordered from Promo Cell (Heidelberg, Germany). Cells had been cultivated from skin samples of the temple taken during plastic surgery at a 40 year-old, male patient. After establishing a primary cell culture the cells were frozen in serum-free freezing medium and sent cryopreserved (500,000 cells in 1ml). A ready-to-use HFDPC Medium and

Supplement Kit (Promo Cell, Heidelberg, Germany), containing Basal Medium, FCS (fetal calf serum), basic FGF and Insulin was additionally ordered to cultivate the cells. For sub cultivation served a kit containing HBSS (HEPES buffered saline solution), trypsin/EDTA solution and neutralizing solution (Promo Cell, Heidelberg, Germany).
Cells were cultivated in T-75 flask with 15ml Medium at 37°C in a humidified atmosphere of 5% CO_2 and sub-cultivated at 85% confluence. Collagen coating was not executed because it was not recommended by the supplier. Cells were frozen and stored at -80°C after each splitting that was up to the sixth passage. For the experiment the cells from third passage were given into 12-well plates (ca. $3.4x10^4$/well) and cultivated till 85% confluence. Cells were cultured in triplicates with 0h (control-treatment), 10nM and 100nM stanozolol groups on each plate, sampling took place 0h (control-time), 6h, 24h and 72h
For treatment, steroids were removed from FCS, as described by Darbre et al. (1983).

RNA extraction and RNA quality
Total RNA was isolated from HFDPC cells using TriFast (peqLab Biotechnologie GmbH, Erlangen, Germany). The standardized protocol from the supplier was used for the extraction. The principal of this protocol was phenol/chloroform extraction for total RNA. Extracted RNA was dissolved in 10 µl RNAse free water. To quantify the amount of total RNA extracted, optical density (OD) was measured with the photometer (Eppendorf Biophotometer, Hamburg, Germany) for each sample.
RNA integrity and quality control was performed with the Bioanalyzer 2100 (Agilent Technology, Palo Alto, USA). For sample analysis eukaryotic total RNA Nano Assay (Agilent Technology) was taken and the RNA Integrity Number (RIN) served as RNA quality parameter (Fleige et al., 2006, Schroeder et al., 2006). In order to the expected high quality of the total RNA from cell cultures, only a set of selected samples from the 6h treatment was measured as reference.

Selection of target genes
Target genes (TG) were selected according to their possible role in the anabolic pathways in the hair follicle. Following TG were chosen as markers by screening the respective literature. Because of the limited total RNA amount, TG were taken for analysis that seemed to be highly influenced by steroids and could show possible gene expression regulations.
Factors of **apoptosis regulation** were Fas receptor ligand (FasL), Fas receptor (FasR), Caspase 8 and the anti-apototic factor Bcl-2. Androgen receptor (AR) and the estrogen receptors (ERα, ERβ) were summarized in the **steroid receptor group**. The **growth factor group** included the insulin like growth factor receptor (IGF1R) and growth hormone receptor

(GHR). Fibroblast growth factor 2 (FGF2) and keratinocyte growth factor (FGF7) were summarized in the **hair cycle factor group**. 5α-Steroidreductases (SRD5A1, SRD5A2) represented the **enzyme group**.
Ubiquitin (UBQ), glycerinaldehyde-3-phsophate-dehydrogenase (GAPDH) and β-actin (ACTB) were taken as reference genes for relative quantification method.

Primer design and testing

All primers were designed using published nucleic acid sequences of "Ensembl Genom Browser" (http://www.ensembl.org) and NCBI (www.ncbi.nlm.nih.gov). Primer design and optimization was done with primer design program primer 3 (http://frodo.wi.mit.edu/) with regard to primer dimer formation, self-priming formation and primer annealing temperature at 60°C. Designed primers were ordered, synthesized and shipped by MWG Biotech (Ebersberg, Germany). Primer testing was performed with a pooled RNA sample of several hair samples, as positive control and RNAse free water as negative control (Qiagen, Hilden, Germany) for each primer set. Generated PCR products were checked for length and primer dimmer formation by an agarose gel electrophoresis. All primer sequences, their amplicon size and annealing temperatures are illustrated in Table 1.

Real-time qRT-PCR

Quantitative real-time RT-PCR was performed at the Rotor Gene 6000 (Corbett Life Science, Sydney, Australia) using SuperScript III Platinum SYBR Green One-Step qPCR Kit (Invitrogen, Carlsbad, USA) by using standard protocol of the supplier. Samples were diluted to 10ng/µl. For mRNA protection, 50ng tRNA in 5µl (Invitrogen, Carlsbad, USA) were added to each sample. 38ng of total RNA were taken for one PCR reaction in a total volume of 10µl. Threshold cycle (Ct) and melting curves were acquired by using the *"quantitation" and "melting curve"* program of the Rotor-Gene 6000 analysis software.
Only genes with clear and single melting peaks were taken for further data analysis. Samples with irregular melting peaks were excluded from the calculation. All samples were baseline corrected and threshold was set manually, using same threshold levels for one gene in all samples.

Data Analysis and Statistics

Data were processed applying relative quantification method comparable to the ΔΔCt-method ($2^{-\Delta\Delta Ct}$) (Livak et al., 2001). Expression changes are shown as relative up- or down-regulation normalized by three internal reference genes. For normalization of target gene expression the arithmetic mean (AM) of the following reference genes (RG) were taken: (RG1) GAPDH, (RG2) ACTB and (RG3) UBQ. The mean values served as reference gene index (RGI). RG

were excluded when showing significant regulation by treatment or time. This value was then taken to do normalization and calculate the ΔCt by subtracting the Ct of the RG-Index (RGI) from the Ct of the target gene (TG).

RGI (cell culture 1) = AM (Ct_{ACTB};Ct_{GAPDH};Ct_{UBQ})

RGI (cell culture 2) = AM (Ct_{GAPDH};Ct_{UBQ})

$$\Delta Ct = Ct_{(RGI)} - Ct_{(TG)}$$

In a second step the normalized gene expression values were set in relation to the control group (ΔΔCt).

$$\Delta Ct_{(control/treatment)} = \text{mean } \Delta Ct \text{ value of control treatment}$$

$$\Delta\Delta Ct = \Delta Ct_{(treatment)} - \Delta Ct_{(control/treatment)}$$

To show up- and down-regulations of the TG the ratio was calculated provided that DNA was doubled per cycle ($2^{\Delta\Delta Ct}$).

$$\text{Ratio} = 2^{\Delta\Delta Ct}$$

Regulations over treatment time were calculated using a 2-Way-Anova with SigmaStat Software. In Excel (Microsoft, USA) ΔΔCt was calculated and statistical analyses were done by using the t-test. All data are illustrated by means±standard deviation (SD).

Results

RNA concentration and RNA integrity

Samples of the cell culture 2 showed a slightly higher mean RNA yield (832±209.9ng) compared to the samples of the cell culture 1 (767.9±225.2ng). For the 6h treatment samples average RIN values were quite similar in cell culture 1 (8.8±0.5) and in cell culture 2 (8.6±2.9).

Primer testing and PCR

From 19 designed and tested primer pairs three genes did not showed satisfactory PCR results, concerning verifiability, melting curve analysis and in agarose gel electrophoresis. Therefore, FasL, ERα and ERβ were excluded from analysis. All other genes were accepted for further calculations.

Gene regulation under Stanozolol treatment

Because the reference gene ACTB showed a significant regulation under stanozolol treatment in cell culture 2, only GAPDH and UBQ were taken for RGI calculation.

Gen expression analysis within one treatment time point ($\Delta\Delta$Ct)

In both cell cultures AR showed a significant down regulation after 24h and FasR an up-regulation after 6h of stanozolol treatment. In cell culture 1 the regulation of AR was seen in the 1nM concentration (p=0.047), in cell culture 2 in the 100nM concentration (p=0.014). FasR was up-regulated at 6h for 10nM concentration in cell culture 1 (p=0.023) and cell culture 2 (p=0.049). Additionally FGF7 was down-regulated at 0h (100nM, p=0.009, 10nM, p=0.046) and at 6h (100nM, p=0.03) in cell culture 1, in cell culture 2 SRD5A2 (6h, 100nM, p=0.028) and FGF2 (0h, 100nM, p=0.010) showed a significant down-regulation (figure 1, figure 2).

Gen expression over treatment time (2-Way-Anova)

In the 2-Way-Anova one significant combination of Caspase 8 to treatment concentration and treatment time could be calculated in cell culture 1. Caspase 8 in the 1nM concentration was significantly different from all other concentrations and significant differences between 0h to 6h and 0h to 48h could be measured for this gene.

Discussion

In the present study it was tested weather the AAS stanozolol induces specific gene expression regulations in HFDPC. Taking the indicated target genes, first potential biomarkers for an expression pattern could be identified. As feasibility study the experiments were done in cell culture to identify a set of target genes possibly interesting for further analysis in human hair follicle samples.

As expected, all cell culture samples showed a very high RNA quality, a precondition for good qRT-PCR results.

Significant regulations over the time period of treatment were supposed to show trends in gene regulations. The separation in different functional groups could help to identify gene dependent pathways in the hair papilla. Significant influences of stanozolol on gene expression could be shown up to an incubation time of 24h. After this time the xenobiotic steroid seems to have less influence on gene expressions. This kind of time dependent gene expression regulations were also shown in other studies (Black et al. 1992, Schönfelder and Einspanier, 2003). Most regulations could be seen at 10nM and 100nM. In both cell cultures AR and FasR showed the same gene expression characteristics under stanozolol treatment at the same time point of treatment. FasR additionally showed comparable regulations at the same hormone concentration. This makes AR and FasR to the first biomarkers for a gene expression pattern of stanozolol. This could show a first gender specific difference because in the male cells the effects of the treatment seem to induce regulations earlier (6h) than in the female samples (24h) (Melchert et al., 1992).

Next to the gender specific effects on gene regulation also the growth phase of the hair follicle could influence the results. A first hind for this assumption could be the different regulations of FGF2 and FGF7 in the untreated samples in both cultures. FGF7 is known to induce the growth phase (anagen phase) in the hair follicle, whereas FGF2 could be taken as its antagonist by inhibiting the morphogenesis (Stenn and Paul, 2001). In cell culture 1 FGF7 was significantly down-regulated what would show that these cells were taken from a hair follicle in the catagen phase. In cell culture 2 FGF2 was significantly down-regulated, indicating the anagen phase. Anyhow the significant regulation of FGF2 was a surprise because several studies identified this factor in the hair follicle but not in the hair papilla (Mitusi et al., 1997; Ota et al., 2002; Schlake et al., 2005).

The significant regulation of FasR and Caspase 8 in cell culture 1 showed first influences on metabolic pathways. The regulation of both factors could indicate a possible influence of stanozolol on the extrinsic way of apoptosis (Reiter et al., 2006).

The significant regulation of IGF1-R is supposed to be an effect of the HFDPC medium because this was already analysed in different studies before (Philpott et al., 1994, Weger et al., 2005, Tang et al., 2003).

Conclusion

In this feasibility study first steps toward a new screening method via gene expression analysis in hair follicle were explored. A set of 10 genes was tested in cell culture to identify possible biomarkers and physiological influences of stanozolol in the hair papilla. Factors like gender and growth phase of the hair follicle seem to influence gene expression patterns in the cells. Additionally FGF2 known to be expressed in the hair follicle was identified in the hair papilla. Anyhow AR and FasR can be taken as first potential biomarkers for a stanozolol treatment pattern because they showed same regulations at the same time point in male and female cell cultures.

In the future these measured target genes could be used for further analysis in hair follicle samples taken from athletes to identify possible treatment with anabolic agents. This could be supportive of the existing test methods in doping analysis and could be an additional proof for illegal drug abuse.

References

Anielski P, Thieme D, Schlupp A, Grosse J, Ellendorf F, Mueller RK. Detection of testosterone, nandrolone and precursors in horse hair. Anal Bioanal Chem, 2005, 383(6):903-8.

Ayotte C. Significance of 19-norandrostenedione in athletes´urine samples. Br J Sports Med, 2006, 40:25-29.

Black MA, Lefebvre FA, Pope L, Lefebvre YA, Walker P. Thyroid hormone and androgen regulation of nerve growth factor gene expression in the mouse submandibular gland. Mol Cell Endocrinol, 1992, 84(1-2):145-54.

Catlin DH, Hatton CK, Starcevic SH. Issues in detecting abuse of xenobiotic anabolic steroids and testosterone by analysis of athletes´urine. Clinical Chemistry, 1997, 43;7:1280-1288.

Clasing D, Müller RK. Dopingkontrolle 2006. 4., überarbeitete Auflage. Bundesinstitut für Sportwissenschaften, 2006.

Darbre P, Yated J, Curtis S, King RJB. Effect of estradiol on human breast cancer cells in culture. Cancer Res, 1983, 43:349-354.

Duntas LH, Parisis C. Doping: a challenge to the endocrinologist. A reappraisal in view of the Olympic Games of 2004. Hormones, 2003, 2(1):35-42

Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Mol A Med 2006, 27: 126-139.

Gambelunghe C, Sommavilla M, Ferranti C, Rossi R, Aroni K, Manes N, Bacci M. Analysis of anabolic steroids in hair by GC/MS/MS. Biomed Chromatogr, 2007, Feb 12.

Gleixner A, Sauerwein H, Meyer HHD. Detection of anabolic β_2-adrenoreceptor agonist clenbuterol in human sclap hair by HPLC/EIA. Clinical Chem, 1996, 42(11).

Griggs RC, Kingston W, Jozefowicz RF, Herr BE, Forbes G, Halliday D. Effect of testosterone on muscle and muscle protein synthesis. J Appl Physiol, 1989, 66(1):498-503.

Hartgens F, Kuipers H. Effects of androgenic-anabolic steroids in athletes. Sports Med, 2004, 34(8):513-54.

Hickson RC, Czerwinski SM, Falduto MT, Young AP. Glucocorticoid antagonism by exercise and androgenic-anabolic steroids. Med Sci Sports Exerc, 1990, 22(3):331-40.

Kuhn CM. Anabolic Steroids. Recent Prog Horm Res, 2002, 57:411-34.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001, 25:402- 408.

Melchert RB, Herron TJ, Welder AA. The effect of anabolic-androgenic steroids on primary myocardial cell cultures. Med Sci Sports Exerc, 1992, 24(2):206-12.

Mitusi S, Ohuchi A, Hotta M, Tsuboi R, Ogawa H. Genes for a range of growth factgors and cyclin-dependent kinase inhibitors are expressed by isolated human hair follicles. Br J Dermatol, 1997, 137: 693-698.

Ota Y, Sioth Y, Suzuki S, Ozawa K, Kawano M, Imamura T. Fibroblast growth factor 5 inhibits hair growth by blocking dermal papilla cell activation. Biochem Biophys Res Commun, 2002, 11;290(1):169-76.

Philpott MP, Green MR, Kealey T. Human hair growth in vitro. J Dermatol Sci, 1994, 7 Suppl:S55-72. Review.

Reiter M, Walf VM, Christians A, Pfaffl MW, Meyer HHD. Modification of mRNA expression after treatment with anabolic agents and its usefulness for gene expression-biomarkers. Anal Chim Acta, 2006, 586(1-2):73-81.

Schänzer W. Metabolism of anabolic androgenic steroids. Clinical Chemistry, 1996, 42:7.

Schlake T. FGF signals specifically regulate the structure of hair shaft medulla via IGF-binding protein 5. Development, 2005, 132: 2981-2990.

Schroeder A, Mueller O, Stocker S et al.The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol, 2006, 31(7): 3.

Schönfleder M, Einspanier R. Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle. Biol Repord, 2003, 69 (1):269-277.

Stenn KS, Paus R. Controls of hair follicle cycling. Pharm Rev, 2001, 81 (1).

Tang L, Bernardo O, Bolduc C, Lui H, Madani S, Shapiro J. The expression of insulin-like growth factor 1 in follicular dermal papillae correlates with therapeutic efficacy of finasteride in androgenetic alopecia. J Am Acad Dermatol, 2003, 49(2):229-33.

Thieme D, Grosse J, Sachs H, Mueller RK. Analytical strategy for detecting doping agents in hair. Forensic Science International, 2000, 107:335-345.

Weger N, Schlake T. IGF-1 signalling controls the hair growth cycle in the differentiation of hair shafts. J Invest Dermatol, 2005, 125:873-882.

Table 1: List of used primer pairs, showing the length of the amplicon (nt), the melt temperature (Tm) and the accession number of the sequence.

Figure 1: Significant gene expression regulations in cell culture 1. The ratio was calculated by $2^{\Delta\Delta Ct}$ whereby the control was set 1 (upper control line). Data are depicted in bars + standard deviations. Red asterisks (*) mark the significantly regulated target genes.

Figure 2: Significant gene expression regulations in cell culture 2. The ratio was calculated by $2^{\Delta\Delta Ct}$ whereby the control was set 1 (upper control line). Data are depicted in bars + standard deviation. Red asterisks (*) mark the significantly regulated target genes.

Table 1

Group	Identity		Sequence 5´-3´	Amplicon (nt)	Tm (°C)	Acces. Nr.
enzymes	SRD5A1	FOR	CTT GAG CCA TTG TGC AGT GT	166	58	ENST00000233239
		REV	GCC TCC CCT TGG TAT TTT GT			
	SRD5A2	FOR	TGA ATA CCC TGA TGG GTG GT	181	60	ENST00000233139
		REV	GGA AAT TGG CTC CAG AAA CAT A			
growth factors	GHR	FOR	ATC CAC CCA TTG CCC TCA AC	246	60	NM00163
		REV	ATC TCA CAC GCA CTT CAT ATT CC			
	IGF1R	FOR	CAT TTC ACC TCC ACC ACC AC	151	60	NM000875
		REV	AGG CAT CCT GCC CAT CAT AC			
hair cycle	FGF2	FOR	AGA AGA GCG ACC CTC ACA TC	237	60	M27968
		REV	ACT GCC CAG TTC GTT TCA GT			
	KGF/FGF7	FOR	CCT GAG CGA CAC ACA AGA AG	167	60	M60828
		REV	GCC ACT GTC GCT TCC TTA TT			
apoptosis factors	FasR	FOR	TTC TGC CAT AAG CCC TGT CC	174	60	NM000043
		REV	CCA CTT CTA AGC CAT GTC CTT C			
	bcl2	FOR	GAG GAT TGT GGC CTT CTT TGA G	170	60	NM000633
		REV	ACA GTT CCA CAA AGG CAT CCC			
	Caspase 8	FOR	TGG CAC TGA TGG ACA GGA G	230	60	NM001228
		REV	GCA GAA AGT CAG CCT CAT CC			
steroid receptors	AR	FOR	TTG TCC ATC TTG TCG TCT TCG G	237	60	L29496
		REV	TGT CCA GCA CAC ACT ACA CC			
reference genes	UBQ	FOR	TGA AGA CTC TGA CTG GTA AGA CC	128	60	NM021009
		REV	CAT CCA GCA AAG ATC AGC CTC			
	GAPDH	FOR	GAA GGT GAA GGT CGG AGT CAA	233	60	NM002046
		REV	GCT CCT GGA AGA TGG TGA TG			
	ACTB	FOR	AGTCCTGTGGCATCCACGAAAC	78	60	NM01101
		REV	GCAGTGATCTCCTTCTGCATCC			

Figure 1

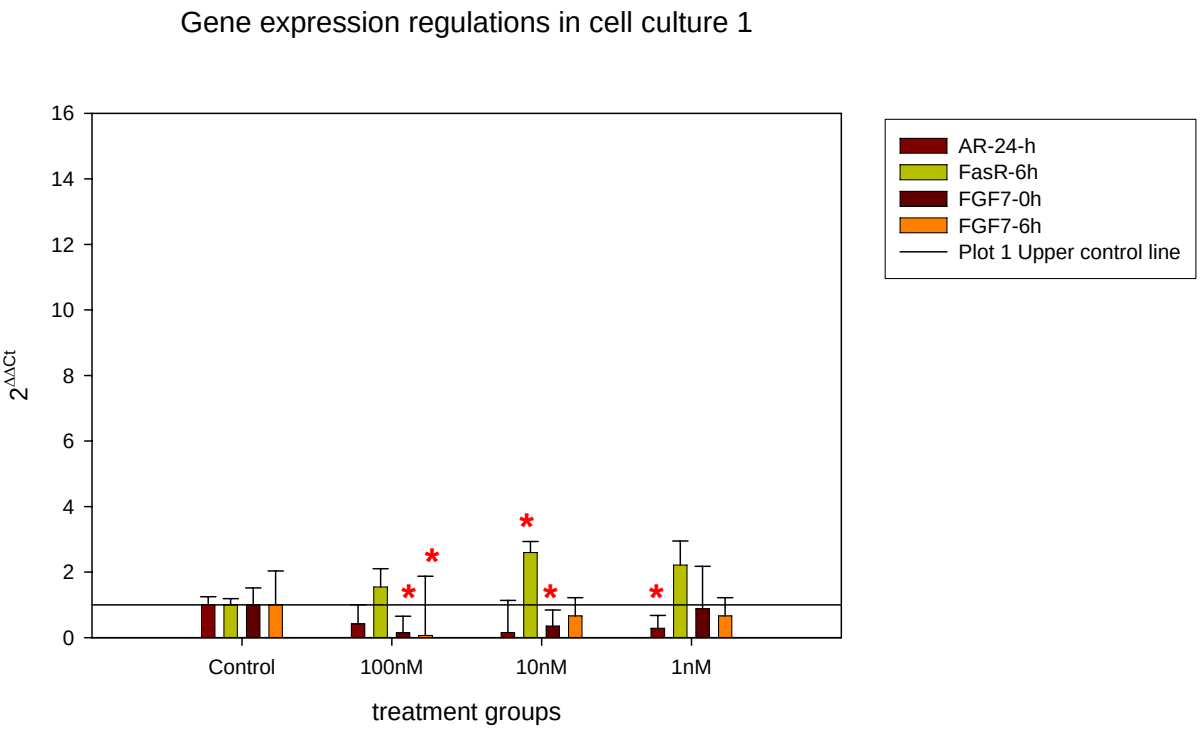
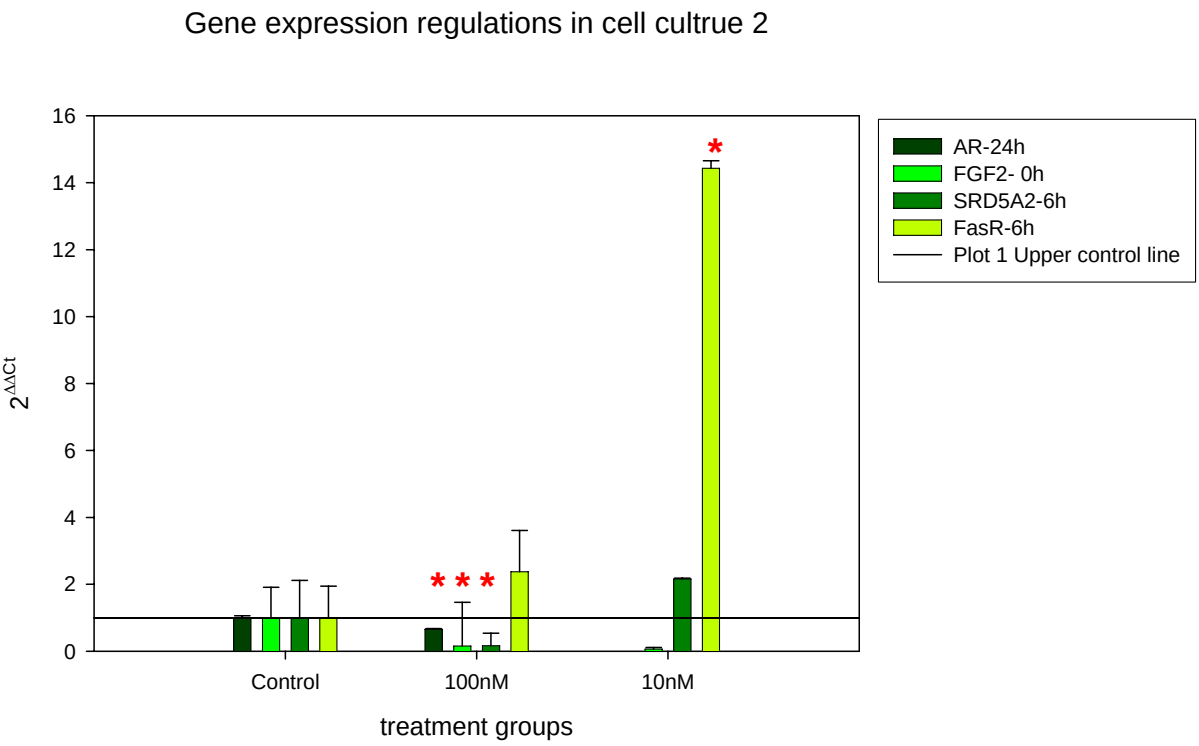

Gene expression regulations in cell culture 1
16
14
12
10
8
6
4
2
0
2^ΔΔCt
Control
100nM
10nM
1nM
treatment groups
AR-24-h
FasR-6h
FGF7-0h
FGF7-6h
Plot 1 Upper control line

Figure 2


Gene expression regulations in cell cultrue 2
16
14
12
10
8
6
4
2
0
2ΔΔCt
* * *
*
Control
100nM
10nM
treatment groups
AR-24h
FGF2- 0h
SRD5A2-6h
FasR-6h
Plot 1 Upper control line

Appendix V

Monitoring gene expression in muscle tissue of *macaca fascicularis* under the influence of testosterone and SARM

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

Physiology Weihenstephan
Technische Universität München
Weihenstephaner Berg 3
85354 Freising
Germany

Corresponding author:
Martina Reiter
Physiology Weihenstephan
TU München
Weihenstephaner Berg 3
85354 Freising
Tel.: ++49-8161-713867
Fax: ++49-8161-714204
martina.reiter@wzw.tum.de

Abstract

The focus of this study was to evaluate data on the gene expression profiles induced by testosterone and a Selective Androgen Receptor Modulator (SARM, TAP Pharmaceutical Products Inc., Lake Forest, USA) in androgen sensitive muscle tissue in order to get better understanding on the molecular mechanisms of action and to identify biomarkers for SARM function in primate organs.

24 male cynomologous monkeys were separated in four groups, a testosterone group, a SARM1 and SARM10 group and a control group, each consisting of 6 animals. The testosterone group was treated i.m. with 3.0 mg/kg Testostoviron®-depot-250 (Schering, Berlin, Germany) every two weeks; the SARM1 and SARM10 group with 1 mg/kg or 10 mg/kg SARM LGD2941 daily, and the control group stayed untreated.

Muscle biopsies from m. quadriceps and m. triceps were collected at three timepoints, baseline timepoint before SARM application (control) and on day 16 and finally on day 90 of treatment.

Thirty candidate genes were selected according to their functionality by screening the actual literature and composed to functional groups: cell cycle, endocrine factors, energy metabolism, muscle fibre proteins, muscle specific transcription factors, protein metabolism and satellite cell biology. Biomarkers were identified as genes regulated from baseline in any of the three treatment groups at day 16 or day 90 using ANOVA with baseline defined as contrast group. From 23 tested candidate genes, 3 were significantly regulated in m. quadriceps after 90 day treatment, in m. triceps no significant differences could be identified. As first biomarkers CTSL, CAPN3 and IGFBP3 could be identified and first physiological differences between control and treatment samples were discovered.

Both the testosterone and the SARM LGD2941 seem to have same effects after 90 day treatment, so that a longer-term therapy with theses substances would be recommended.

Keywords: testosterone, selective androgen receptor modulator, expression profiling, candidate gene approach, qRT-PCR

1. Introduction

Due to demographic changes in our population sarcopenia and frailty become major Ctics in health care profession. Already 30% of those over 60 years are affected. Sarcopenia - the loss of skeletal muscle mass and strength, in both man and women results in high incidence of accidental falls and can compromise quality of life. Chronic, age related afflictions like osteoporosis are linked to sarcopenia. The decrease in the production of hormones such as testosterone, estradiol, growth hormone, insulin like growth factor 1 and general decline in muscle protein turnover, as well as neuromuscular alterations, are the major reasons for the appearance of frailty. This results in weakness, impaired mobility and poor endurance, factors that lower independence and reduce the quality of life [5, 19, 26, 28].

The muscles are affected by the lower endogenous anabolic hormone levels and muscle protein turnover. Studies have shown that muscle strength, muscle cross-sectional area decreases and hypotrophy arises [7, 8, 16]. Androgen replacement therapies are the first-line therapies for osteoporosis and frailty, not only for men also for women [1, 24]. Particularly for women the therapeutic uses of androgens do not only have beneficial effects because numerous side effects exist [10, 11, 12, 13, 18].

A promising alternative for androgen replacement therapies, including osteoporosis and frailty, could be the discovery of selective androgen receptor modulators (SARM). SARM have the ability to mimic the central and peripheral androgenic and anabolic affects of testosterone without having the negative side effects of the natural hormone [6]. Further positive properties of now investigated SARM, like the oral activity, increase in muscle mass and strength and increase in fat-free mass, are important factors for its use as medicament [20, 22, 29].

Applying anabolic agents, changes in the biochemical pathways are notified in the organs and tissues. Expression of specific enzymes, receptors and cytokines can be activated or suppressed on the cellular mRNA expression level. Using appropriate specific and sensitive quantification methods, such as the quantitative real-time RT-PCR, even minute expression changes are measurable as a difference in the respective mRNA.

The aim of this study was to show how a SARM in comparison to testosterone may act in muscle tissues via mRNA expression of different candidate genes. Sufficient plurality of regulated candidate genes may then serve as biomarkers to show molecular, myogenic effects of the treatments.

2. Materials and Methods

2.1. Animal experiment

The animal husbandry and tissue collection were done by Covance Laboratories GmbH (Münster, Germany). Groups of 6 male cynomologous monkeys (*macaca fascicularis*) were either injected i.m. with 3.0 mg/kg Testostoviron®-depot-250 (Schering, Berlin, Germany) in 14 days periods (Testo), or orally treated with 1 mg/kg (SARM1) or 10 mg/kg (SARM10) LGD2941 (TAP Pharmaceutics, Lake Forest, USA) every day. The control group without any treatment consisted of 6 animals. All monkeys were 5-6 years old and had an average body weight of 6 kg.

2.2 Sample collection

Muscle biopsies of *m. quadriceps* and *m. triceps* were taken at three time points. Control samples were taken without prior treatment. Further samples were taken at day 16 and day 90 of treatment. Biopsy samples were placed in kryotubes before freezing in liquid nitrogen and stored at –80°C until RNA-extraction.

2.3 Total RNA extraction

50 mg of muscle biopsies on baseline time point (control) and 100 mg on the time points day 16 and day 90 were colleted. Frozen tissue was homogenized in guanidinium thiocyanate buffer to destroy RNase activity. For the extraction of the RNA a TriFast clean protocol was used (PeqLab Biotechnologies, Erlangen, Germany). The principal of this protocol was phenol/chloroform extraction for total RNA. To quantify the amount of total RNA extracted, optical density (OD) was measured with the photometer (Eppendorf Biophotometer, Hamburg, Germany) for each sample. All RNA stem solutions were diluted to 100 ng/μl for Bioanalyzer analysis and to 1 ng/μl for PCR analysis.

2.4 Analysis of RNA quality

RNA integrity and quality control was done by capillary electrophoresis in a Bioanalyzer 2100 (Agilent Technology, Palo Alto, USA). Eukaryotic total RNA Nano Assay algorithm was taken for sample analysis and the RNA Integrity Number (RIN) was used to indicate

RNA quality [14, 23]. For optimal RNA quality biopsy storage in kryotubes is recommended.

2.5 Selection of candidate genes

Candidate genes, factors and enzymes were selected if assumed to play an important role in the myogenic pathways of the analysed tissues. Following 30 candidate genes (TG) were chosen by screening literature.

Muscle fibre proteins: myosin (MYHC-2x) and α-actin (ACTA1); *muscle specific transcription factors*: myogenin and myogenic differenciation factor (MyoD); *protein metabolism group:* cathepsin B (CTSB), cathepsin L (CTSL), calpain3 (CAPN3) and tyrosin-amino-transferase (TAT); *factors of cell cycle regulation*: cyclin-dependent kinase 2 (CDK2), Cyclin A, Cyclin D1 and myostatin (GDF8); *endo- and paracrine group*: androgen receptor (AR), estrogen receptors (ERα, ERβ), growth hormone receptor (GHR), glucocorticoid receptor (GR), insulin like growth factor 1 (IGF1), insulin like growth factor receptor 1 (IGF1R), insulin receptor (IR, transcript1), insulin like growth factor binding proteins (IGFBP3, IGFBP4); *energy metabolism group*: hexokinase (HK), lactat-dehydrogenase (LDH), creatinkinase (CK), glucose transporter 4 (GLUT4) and glycogen phorphorylase (PYGM); *satellite cell group*: myogenic factors (Myf5, Myf6) and m-cadherin (CDH15).

As *reference genes* (RG) beta-Actin (ACTB), Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) Cyclophilin A (PPIA), Tyrosin3/trypCthan5-monooxygenase activation protein (YWHAZ), Ubiquitin C (UBC) and 18S RNA primers were tested.

2.6 Primer design and primer testing

Primers were designed using published nucleic acid sequences of the human genome GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). PPIA and ACTB were taken from the Human Endogenous Control Gene Panel Kit (TATAA Biocenter, Göteborg, Sweden). Primer design and optimization was done using primer design program of MWG (MWG, Ebersberg, Germany) with regard to primer dimer formation, self-priming formation and primer annealing temperature (60°C). Newly designed primers were ordered and synthesized at MWG Biotech (Ebersberg, Germany) and Invitrogen (Karlsruhe, Germany). Primer testing was performed on four muscle samples and a negative control (RNAse free water) for each primer set.

2.7 One-step quantitative RT-PCR

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 manufacture.

Crossing points (CT) and melting curves were acquired by using the *"comparative quantitation" and "melting curve"* program of the Rotor-Gene 3000 analysis software. CT is the equivalent nomenclature of Cycle of threshold used in Rotor-Gene software.

Only genes with clear melting curves were taken for further data analysis. Samples with irregular melting peaks were excluded from the calculation.

2.8 Data Analysis and Statistics

Statistical descriptions of the expression data as well as statistical tests were produced with SAS v. 9.1.3 for Windows. The raw data was the CT values obtained from each qPCR sample. Each qPCR sample was associated with a biopsy sample whereas form each experimental animal two biopsy samples were analysed. Since the 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 biopsy samples. The obtained mean CT values were then translated to normalized expression quantities using all three reference genes in a form of normalization index. The normalization index was calculated as an arithmetic mean of the CT values of the three reference genes:

$$\text{reference index} = \text{mean}\,(CT_{PPIA}, CT_{ACTB}, CT_{YWHAZ}) \quad (1)$$

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

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

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, generating the expression ratio R as:

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

The expression ratio R was then analysed statistically. The Box-whisker plot was constructed to facilitate visual screening of regulated genes (Figure 1-4).
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 tests were produced.

3. Results

3.1 RNA quality of muscle tissue

The mean RIN value of control samples was 6.5 for the m. quadriceps and 6.9 for the m. triceps samples, representing a total RNA of average quality. The m. quadriceps samples of day 16 had a mean RIN of 7.1, the m. triceps of 7.6. In day 90 m. quadriceps samples showed a mean RIN of 6.9, m. triceps samples of 7.6.
Control samples showed a higher 5S RNA peak for all samples which may be a hint for partly degraded total RNA. Because control biopsy samples were directly frozen in liquid nitrogen, it is supposed that large RNA was partly damaged but the smaller 5S RNA remained intact. In samples given into kryotubes before freezing in liquid nitrogen, large RNA was more intact.

3.2 Primer pairs and primer testing

Based on the human genome, primer pairs for 30 TG and 6 RG were developed, 23 TG and 3 RG were successfully running in qRT-PCR (Table 1). Until now nearly no gene sequences from *macaca fascicularis* exist and because these sequences seem to be different from human, some primer pairs did not anneal.

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.
In m quadriceps significant differences between the control and treatment could be identified after 90 day treatment, including CTSL ($p=0.0138$), CAPN3 ($p=0.0625$) and IGFBP3 ($p=0.0280$) (Figure 1, 2, 3).
There were no significantly regulated genes found in m. triceps. Possible effect of outliers was investigated and could not be excluded. Only the three significantly regulated genes on the time point 90 days in m. quadriceps are hence discussed further. Surprisingly the expression ratio R deviated from the value one in the control group, showing thus endogenously induced up-regulation throughout the growth period of the animals. This

conclusion is supported by the fact that no pronounced regulation was found on the time point 16 days.

The control group showed also high variability as compared to the treatment groups as indicated by the box-whisker plot. This reflects the natural variability of the non-induced expression in each studied subject. In contrast to the control group, in the treated groups that showed lower R also smaller variability was observed, possibly supporting the evidence for induced suppressing effect on the individual’s expression.

4. Discussion

Aging is associated with many different processes in the body such as muscle atrophy which corresponds to a loss of muscle tissue caused by decreases in fibre size and/or a reduction in the number of fibres. So far it has not been understood if this effect depends on a reduced availability of mRNA, on an energy deficit due to a loss of mitochondria or on a decrease in nitrogen supply. [2, 27]. For therapies, anabolic steroids and SARM are investigated to antagonize these effects. To get more information about factors that influence these muscle reducing pathways and the effect of anabolic agents on muscle metabolism, gene expression of selected genes was measured using real-time qRT-PCR. The RNA quality of all samples was sufficient to get satisfactory results in qRT-PCR.

Only a few gene sequences of *macaca fascicularis* were available in public gene banks so the gene sequences of *homo sapiens* and *macaca mulatta* were used for primer pair design. In spite of this problem 23 candidate genes could be successfully amplified.

Only gene expression regulations with comprehensible and coherent results are discussed to avoid speculations. Significant differences between the control and the treatment groups could only be seen after 90 day treatment.

In m. quadriceps the control samples already showed an expression with a high statistical spread in the significantly regulated candidate genes what mirrors natural differences between gene expressions in the individuals. This spread reduced in the treatment groups, showing a suppression of the individual expression differences induced by the treatment. This effect could not only be seen in the significantly regulated genes, also IR in m. quadriceps showed larger spread in the control than in the treatment groups (Figure 4). This difference in spread was strongly pronounced in the testosterone group, indicating an inhibitory effect of testosterone on IR expression.

To identify physiological differences between the control and the treatment, only the significantly regulated IGFBP3, CTSL and CAPN3 are discussed: In skeletal muscle cell lines four IGFBPs are released: IGFBP-2, -4, -5, -6. IGFBP3 mainly acting as major carrier of the IGFs in the circulation. Many studies have also shown an inhibitory effect of IGFBP3 on IGF1 and IGF2, Due to its higher affinity to IGF1, it can also prevent IGF1 - IGF1-R interactions [3, 15, 17, 21]. In this study, IGFBP3 was down-regulated in the treatment samples what could show a switch-off effect of the inhibiting IGFBP3. Even if IGF1 was not significantly up-regulated, a blockage of IGFBP3 could increase its functionality in the organism.

In the protein metabolism of the muscle CTSL is induced early in the catabolic states and an increase of CTSL expression also characterizes muscle wasting. Anabolic steroids like testosterone increase protein retention and decrease protein catabolism [4]. In this study the significant down-regulation of CTSL in the treatment confirms this protein retention effect.

CAPN3 is a member of the three distinct calpains expressed in the muscle. The ubiquitous calpains 1 and 2 and CAPN3 are members of the Ca2+-activated cysteine proteases but CAPN3 is known to show inverse regulations compared to the ubiquitous calpains. Many studies have proofed that a decrease of CAPN3 in directly related to muscle wasting [2, 9]. No studies exist that show an effect of testosterone on calpain expression. In this study CAPN3 was significantly down-regulated by the treatment as compared to the control. Such a down-regulation of CAPN3 could also be seen in conditions related to disuse and denervation of the muscle, an age-related phenomenon [25]. In this study the daily agitation of the monkeys was not documented but the animals were kept in cages which might have induced disuse of the muscle and explain the down-regulation of CAPN3 in this study.

Conclusion

In this study both the anabolic steroid testosterone and the SARM LGD2941 showed effects on the gene expression in the muscle tissues. Significant influences of the treatments appeared after 90 days only indicating that the differences between the control group and the treated groups is rather based on suppression of long term natural development of the studied factors. First influences of the treatments on the protein metabolism and the muscle growth could be seen via the significant expression of CTSL, CAPN3 and IGFBP3. These genes can be taken as the first biomarkers for a gene expression pattern of anabolic steroids.

Acknowledgments

We are indebted to TAP Pharmaceuticals Inc. Lake Forest, USA who initiated and supported this study.

References

1. Bhasin S, Bagatell CJ, Bremner W.J et al. Therapeutic perspective: Issues in Testosterone Replacement in Older Men. JCEM 1998, 83 (10): 3435-3436.

2. Bartoli M, Richard I. Calpains in muscle wasting. Int J Biochem Cell Biol 2005, 37 (10): 2115-2133.

3. Baxter RC. Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. Am J Physiol Endocrinol Metab 2000, 278 (6): E967-E976.

4. Bechet D, Tassa A, Taillandier D et al. Lysosomal proteolysis in skeletal muscle. J Biochem Cell Biol 2005, 37: 2098-2114.

5. Bross R, Storer T, Bashin S. Aging and Muscle loss. TEM 1999, 10(5): 194-198.

6. Chen F, Rodan GA, Schmidt A. Development of selective androgen receptor modulators and their therapeutic applications. Zhonghua Nan Ke Xue. 2002, 8(3):162-8.

7. D'Antona G, Pellegrino MA, Adami R et al. The effect of ageing and immobilization on structure and function of human skeletal muscle fibres. J Physiol 2003, 552(2): 499-511.

8. D'Antona G, Lanfranconi F, Pellegrino MA et al. Skeletal muscle hypertrophy and structure and function of skeletal muscle fibres in male body builders. J Physiol 2006, 570(3): 611-627.

9. Dargelos E, Moyen C, Dedieu S. Development of an inducible system to assess p94 (CAPN3) function in cultured muscle cells. J Biotech 2002, 96: 271-279.

10. Davis S. Androgen Replacement in Women: A Commentary. JCEM 1999, 84(6): 1886-1891.

11. Davis SR. The therapeutic use of androgens in women. J St Biochem Mol Biol 1999, 69:177-184.

12. Deschenes MR. Effects of aging on muscle fibre type size. Sports Med 2004, 34(12): 809-24.

13. Doherty TJ. Invited review: aging and sarcopenia. J Appl Physiol 2003, 95(4): 1717-27.

14. Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Mol A Med 2006, 27: 126-139.

15. Florini JR, Ewton DZ, Coolican SA. Growth hormone and the insulin-like growth factor system in myogenesis. Endocr Rev 1996, 17(5): 481-517.

16. Frontera WR, Hughes VA, Fielding RA. Aging of skeletal muscle: a 12-yr longitudinal study. J Appl Physiol 2000, 88: 1321-1326.

17. Jones JI, Clemmons DR. Insulin-Like Growth Factors and Their Binding Proteins: Biological Actions. Endocrine Reviews 1995, 16(1): 3-34

18. Lopez FJ. New approaches to the treatment of osteoporosis. Curr Opin Chem Biol 2000, 4 (4):383-393.

19. Muller M, Grobbee DE, Thijssen JHH. Sex hormones and male health: effects on components of the frailty syndrome. Trends Endocrinol Metabol 2003, 14(6): 289-296.

20. Negro-Vilar A. Selective Androgen Receptor Modulators (SARMs): A Novel Approach to Androgen Therapy for the New Millennium. JCEM 1999, 84(10): 3459-3462.

21. Pell JM, Bates PC. Differential actions of growth hormone and insulin-like growth factor I on tissue protein metabolism in dwarf mice. Endocrinology 1992, 130: 1942-1950.

22. Rosen J, Negro-Vilar A. Novel, non-steroidal, selective androgen receptor modulators (SARM) with anabolic activity in bone and muscle and improved safety profile. J Musculoskel Neuron Interact 2002, 2(3): 222-224.

23. Schroeder A, Mueller O, Stocker S et al.The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 2006, 31(7): 3.

24. Snyder PJ, Peachy A, Hannoush P. Effect of Testosterone Treatment on Body composition and Muscle Strenght in Men Over 65 Years of Age. JCEM 1999, 84(8): 2647-2653.

25. Stockholm D, Herasse M, Marchand S, Praud C, Roudaut C, Richard I, Sebille A, Beckmann JS. Calpain 3 mRNA expression in mice after denervation and during muscle regeneration. Am J Physiol Cell Physiol 2001, 280(6):C1561-9.

26. Van den Beld A, Huhtaniemi IT, Pettersson KS et al. Luteinizing Hormone and Different Genetic Variants, as Indicators of Frailty in Healthy Elderly Men, JCEM 1998, 84(4): 1334-1339.

27. Welle S, Bhatt K, Thornton C. Polyadenylated RNA, actin mRNA, and myosin heavy chain mRNA in young and old human skeletal muscle. Am J Physiol 1996, 270(2): E224-9.

28. Yialamas MA, Hayer FJ. Androgens and the ageing male and female. Best Practice and Research, Clin Endocrin and Metabol 2003, 17(2): 223-226.

29. Yin D, Gao W, Kearbey JD. Pharmakodynamics of Selective Androgen Receptor Modulators. JPET 2003, 304(3): 1334-1340.

Legend

Table 1: List of primer pairs used for PCR analysis in muscle tissue.

Figure 1: Significant down-regulation of CTSL between control and treated samples. Box-whisker plot show the expression ratio R between the normalised expression of the timepoint 90 and baseline. The horizontal line inside the box indicates median and the box indicates the interquertile range. The full point indicates mean. The whiskers indicate the maximum and minimum and the squer indicates outliers. *The diamond indicates weak outliers.* The calculation of the outliers is based on the box-whisker rule saying that a outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

Figure 2: Significant down-regulation for CAPN3 between control and treated samples. Box-whisker plot show the expression ratio R between the normalised expression of the timepoint 90 and baseline. The horizontal line inside the box indicates median and the box indicates the interquertile range. The full point indicates mean. The whiskers indicate the maximum and minimum and the squer indicates outliers. *The diamond indicates weak outliers.* The calculation of the outliers is based on the box-whisker rule saying that a outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

Figure 3: Significant down-regulation for IGFBP3 between control and treated samples. Box-whisker plot show the expression ratio R between the normalised expression of the timepoint 90 and baseline. The horizontal line inside the box indicates median and the box indicates the interquertile range. The full point indicates mean. The whiskers indicate the maximum and minimum and the squer indicates outliers. *The diamond indicates weak outliers.* The calculation of the outliers is based on the box-whisker rule saying that a outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

Figure 4: IR in the control group shows a high statistical spread which can not be seen in the treatment groups, especially in the Testo group. Box-whisker plot show the expression ratio R between the normalised expression of the timepoint 90 and baseline. The horizontal line inside the box indicates median and the box indicates the interquertile range. The full point indicates mean. The whiskers indicate the maximum and minimum and the squer indicates outliers. *The diamond*

indicates weak outliers. The calculation of the outliers is based on the box-whisker rule saying that a outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

Table 1

Group	Primer	Sequenz 5´- 3´	bp	Acces. Nr.
muscle fibre proteins	ACTA1 for	CAT GGT CGG TAT GGG TCA GAA	21	NM001100
	ACTA1 rev	CGC GAA GCT CGT TGT AGA AG	20	
	MYHC-2x for	GCA CAT CCA GAG CAG AGA AGA A	22	NM017534
	MYHC-2x rev	GAC AGT GAC ACA GAA GAG ACC T	22	
transcription factors	Myogenin for	ATC ATC TGC TCA CGG CTG AC	20	NM002479
	Myogenin rev	TTT CAT CTG GGA AGG CCA CAG A	22	
protein metabolism	CTSB for	ACAATTCCTACAGCGTCTCC	20	L16510
	CTSB rev	CACCACTTCTGATTCGATTCC	21	
	CTSL for	ACATCCCTAAGCAGGAGAAGGC	22	M20496
	CTSL rev	CCAGCACACCATGATCCATGTC	22	
	CAPN3 for	TCCAGTTCGTCTGGAAGAGACC	22	AY902237
	CAPN3 rev	TGTACGTTGGCAGGCAGTCATC	22	
cell cycle regulation	CyclinD for	AAC AAG CTC AAG TGG AAC CTG G	22	NM053056
	CyclinD rev	CAT GGA GGG CGG ATT GGA AAT	21	
	Myostatin for	AAC TTG ACA TGA ACC CAG GCA C	22	NM005259
	Myostatin rev	ACG GAT TCA GCC CAT CTT CTC	21	
endo- paracrin factors	AR for	TTG TCC ATC TTG TCG TCT TCG G	22	L29496
	AR rev	TGT CCA GCA CAC ACT ACA CC	20	
	ERbeta for	ATG CTC ACT TCT GCG CTG TC	20	NM001437
	ERbeta rev	CAC ACT TCA CCA TTC CCA CTT C	22	
	GHR alpha for	ATC CAC CCA TTG CCC TCA AC	20	NM00163
	GHR alpha rev	ATC TCA CAC GCA CTT CAT ATT CC	23	
	IGF-1R for	CAT TTC ACC TCC ACC ACC AC	20	NM000875
	IGF-1R rev	AGG CAT CCT GCC CAT CAT AC	20	
	IGFBP3 for	CAC AGA TAC CCA GAA CTT CTC C	22	M35878
	IGFBP3 rev	CCA TAC TTA TCC ACA CAC CAG C	22	
	IGFBP4 for	CAA CTT CCA CCC CAA GCA GT	20	NM001552
	IGFBP4 rev	CTG GTG GCA GTC CAG CTC	18	
	IR for	TCC AGA CAG ATG CCA CCA AC	20	NM000208
	IR rev	ATC CAG CTC GAA CAG CTC AC	20	
	IGF1 for	TGG ATG CTC TTC AGT TCG TGT G	22	ENSMMUT03165
	IGF1 rev	TAG AGG GAG TGC AGG AAA CAA G	22	
	GR for	TTC TGC GTC TTC ACC CTC AC	20	AH002750
	GR rev	CTG TCT CTC CCA TAT ACA GTC C	24	
energy metabolism	HK for	CGC ATC TGC TTG CCT ACT TC	20	NM000189
	HK rev	AAC TCT CCG TGT TCT GTC CC	20	
	LDH for	TTC AGC CCG ATT CCG TTA CC	20	NM005566
	LDH rev	CAC CTC ATA AGC ACT CTC AAC C	22	
	CK for	GAC ATC GTC TAC AGT GAA GCC	21	NM001824
	CK rev	AGT TCA AGC TGA ATT TAC AAG CCT G	25	
	PYGM for	ACA TCA ACC CCA ACT CAC TCA C	22	AF066859
	PYGM rev	GCC TTC CCT CCA ATC ATC AC	20	
satellite cell biology	Myf5 for	TGA GAG AGC AGG TGG AGA AC	20	NM005593
	Myf5 rev	GGC AAC TGG AGA GAG AGA AG	20	
	Myf6 for	GCC AAG TGT TTC CGA TCA TTC C	22	NM002469
	Myf6 rev	ACT TCT CCA CCA CTT CCT CC	20	
reference genes	beta-actin for	TATAA Biocenter		
	beta-actin rev	TATAA Biocenter		

	PPIA for	TATAA Biocenter		
	PPIA rev	TATAA Biocenter		
	YWHAZ for	GCA ACC AAC ACA TCC TAT CAG AC	23	NM145690
	YWHAZ rev	TTC TCC TGC TTC AGC TTC GTC		

Figure 1

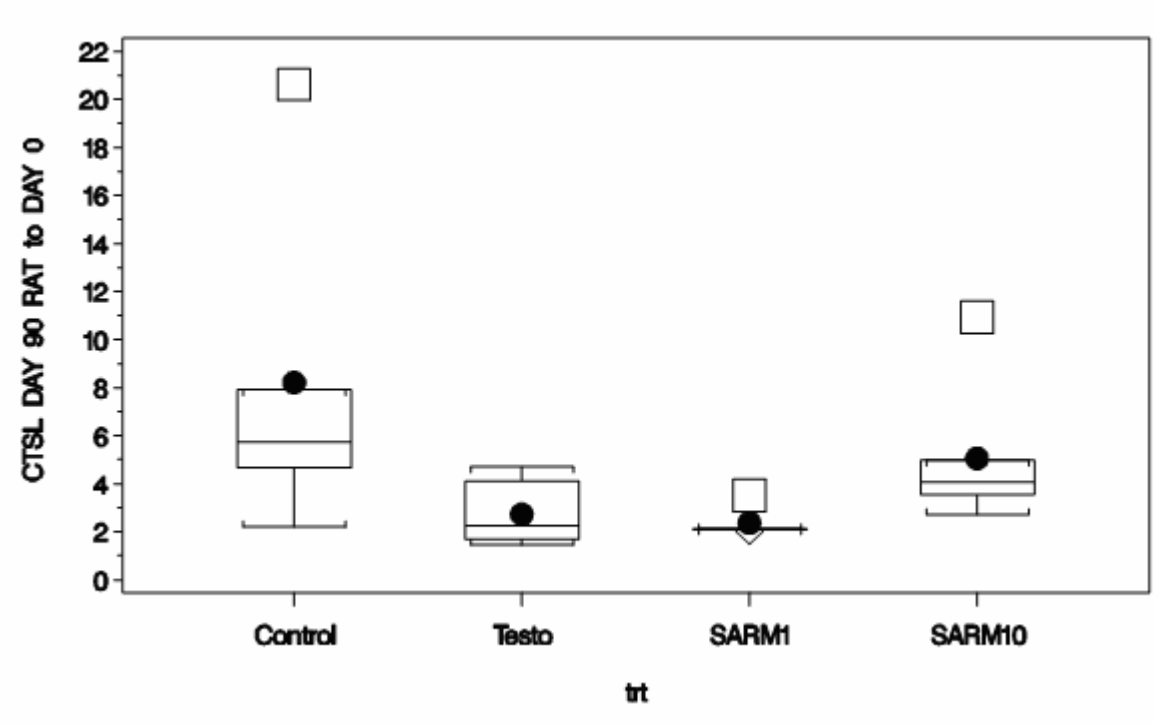

CTSL DAY 90 RAT to DAY 0
22
20
18
16
14
12
10
8
6
4
2
0
Control
Testo
SARM1
SARM10
trt

Figure 2

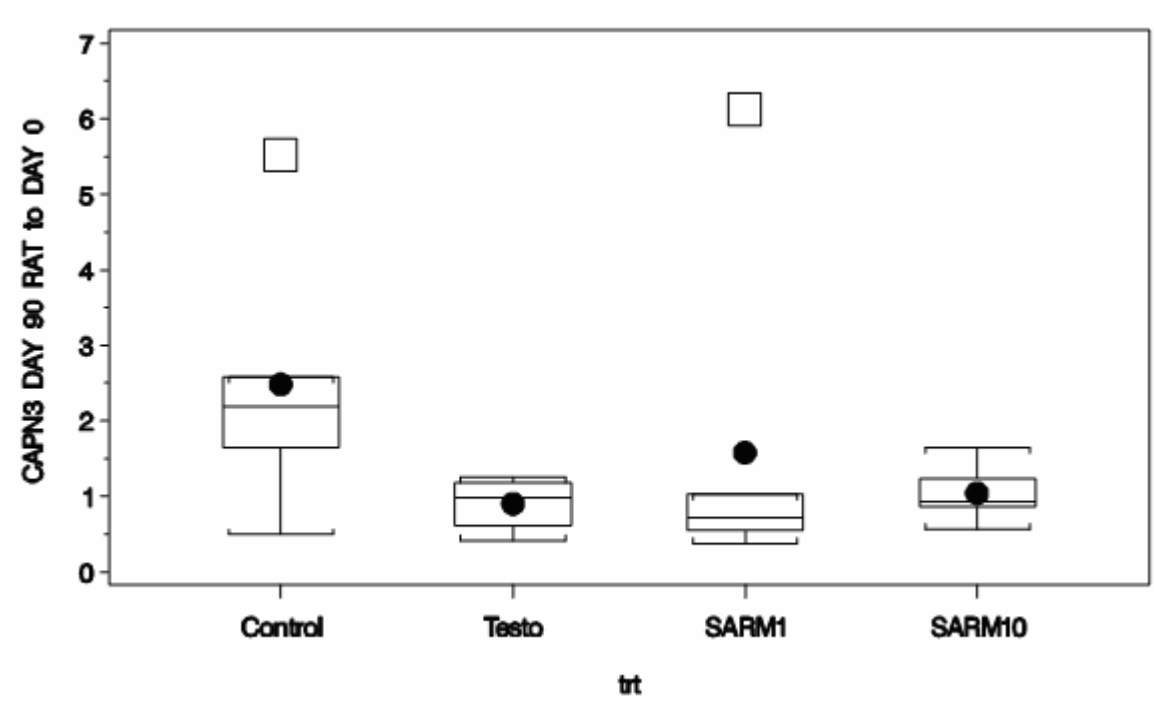

CAPN3 DAY 90 RAT to DAY 0
7
6
5
4
3
2
1
0
Control
Testo
SARM1
SARM10
trt

Figure 3

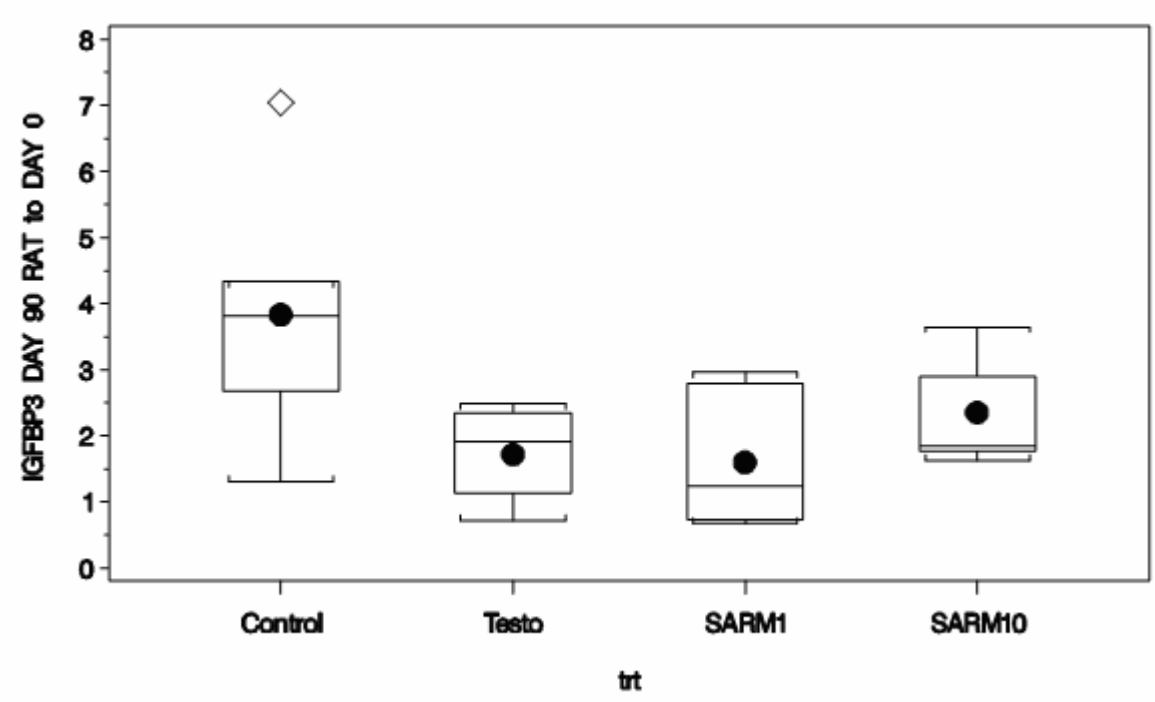
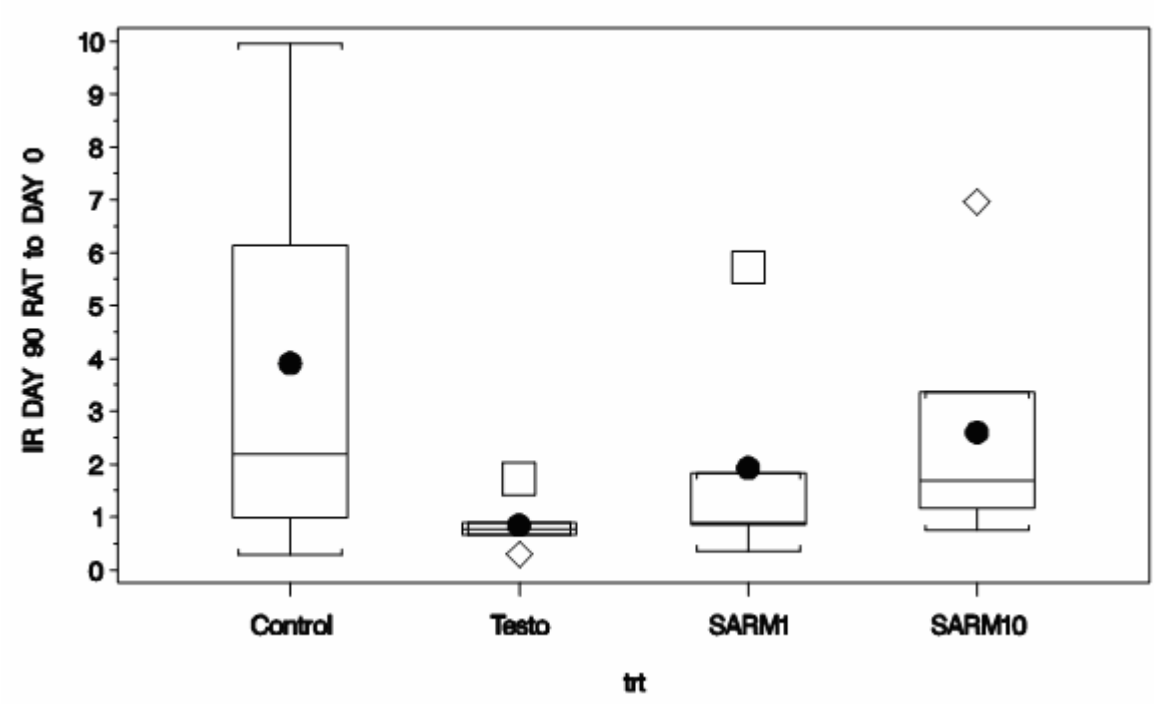

IGFBP3 DAY 90 RAT to DAY 0
8
7
6
5
4
3
2
1
0
Control
Testo
SARM1
SARM10
trt

Figure 4


IR DAY 90 RAT to DAY 0
10
9
8
7
6
5
4
3
2
1
0
Control
Testo
SARM1
SARM10
trt