Research article

Drug Testing and Analysis

Received: 14 April 2011 Revised: 5 August 2011 Accepted: 5 August 2011 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/dta.360

Gene expression profiling in human whole blood samples after controlled testosterone application and exercise

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Doping with anabolic agents is regulated within a number of sports. Testosterone and its functional analogs are popular compounds for increasing muscle mass, physical performance, recovery, and reducing body fat. While routine tests for anabolic drugs exist (e.g. hair, urine, and blood analysis), the aim of the present study is to determine specific gene expression profiles (induced by testosterone and exercise) which may be used as effective biomarkers to determine the use of anabolic drugs. In this study, whole blood samples of 19 male volunteers were analyzed by semi-quantitative real-time polymerase chain reaction (RT-PCR) for gene expression profiles in the context of exercise and transdermal testosterone application (1.5 mg/kg body weight). The hormone application was monitored by urine and saliva analysis for testosterone. Both urinary and saliva levels indicate that transdermal testosterone application leads to an increase of testosterone, especially after exercise. RT-PCR results showed a clear variation in the expression of target genes as well as established housekeeping genes. Only one of the nine common housekeeping genes, cyclophilin b (PPIB), appears to be independent of both exercise and testosterone. Out of 14 candidate genes, five are unregulated; all others were more or less influenced by the mentioned variables. Only interleukin-6 appeared to be exclusively dependent on long-term testosterone application. This study indicates that many genes are not influenced by testosterone alone while exercise modulates gene expression in whole blood samples. As such, exercise must be considered when validating gene expression techniques for doping analysis. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: gene expression; testosterone; exercise; doping analysis; whole blood cell; salivary testosterone; urinary testosterone

Introduction

Anabolic androgenic steroids (AAS) are misused by athletes because of their growth-promoting properties in bone and muscle, together with their ability (at low doses) to improve recovery time and muscle recuperation.[1] The main functions of AAS are to stimulate protein synthesis and an antiglucorticoid effect which increases muscle mass and strength. AAS do not directly improve force or endurance capacity. The anabolic effect of testosterone on the skeletal muscle is mediated through androgen receptor signaling. Testosterone promotes myogenic differentiation of multipotent mesenchymal stem cells and inhibits their differentiation into the adipogenic lineage. Testosterone binding to androgen receptors induces downstream cell differentiation pathways which are included in embryogenesis and cancer development. Singh *et al*. elucidated that testosterone influences several genes of Wnt (*Wingless/Int-1*) cell signaling pathways, including follistatin, which is known to promote myogenic differentiation.[2] They postulate that testosterone-androgen receptor complex interacts with beta catenin, a protein with is downstream implemented into the Wnt signaling pathway. In addition, it is reported that AAS influence the central nervous system and increase motivation and performance.[3–6]

The International Olympic Committee banned the use of synthetic AAS in 1974 by athletes but today steroid doping remains a concern in almost all genres of sport. The World Anti-Doping Agency (WADA) encourages drug testing laboratories to develop methods to detect AAS like testosterone, 17β-nortestosterone and stanozolol, the most frequently found steroids in doping samples.[7,8] Common methods for determining doping are mass spectrometry (MS) based techniques which directly detect the AAS or their metabolites in different tissues like urine, blood or hair samples.

As hormones are detectable over a long time and the samples are easily to collect by a noninvasive manner, hair analysis is becoming an attractive form of doping analysis.[9,10]

The intake of AAS influences the organism in many different ways. It has been reported that hormonally provoked changes in metabolism can be seen on the level of mRNA gene expression. In gene expression studies on heifers, Pfaffl *et al*. have shown that tissue-specific gene expression pattern after a long-term supplementation with several anabolic agents.[11] Here, the uterus has been shown as the most sensitive tissue, indicating the highest changes in gene expression under anabolic treatment. Anabolic agents also show significant gene expression in the liver and

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muscle tissue. Previous studies on dermal papilla cells of human hair roots indicate that several genes, such as the androgen receptor, the Fas receptor, and the fibroblast growth factor-2 are influenced by AAS.[12] Additionally, in a preliminary study on blood cells in rat, the authors have shown that *in vivo* mRNA expression of the androgen receptor (AR) is repressed by exogenous testosterone application and is possibly influenced by chronic exercise.[13] By using transdermal testosterone application, the AR was significantly down regulated after 6 h and 24 h in exercising and non-exercising rats. Furthermore, in exercising rats AR gene expression recovered earlier as in sedentary rats.

Aim of the study

The inability of conventional MS-based techniques in doping analysis to detect structurally novel androgens has led to the requirement for alternative bioassays to identify androgens by their bioactivity. In this context, Akram *et al*. have shown that it is possible to detect nutraceutical-derived steroids by using mammalian *in vitro* androgen bioassays through HEK293 and HuH7 cell lines transfected with the human AR.[14] In concert with receptor-based techniques, the direct modification of gene expression may be a suitable bioassay to determine androgenic activity of anabolic agents. Diel *et al*. have shown that in the liver and in several muscle types of rats the agent desoxymethyltestosterone could be a potent modulator of gene expression of several target genes such as tyrosine aminotransferase, IGF-I and myostatin.[15] A recent study by Paparini *et al*. on human haematopoetic cells has elucidated that nandrolone, IGF-I or growth hormone, significantly and consistently modulated the expression of some candidate genes such as AR, ESR2, PGR, SRD5A1, PPARA, JAK2, EPOR and PGR in a cell specific manner.[16]

Taking this into account, this study aims to validate possible target genes of testosterone application in a preliminary *in vitro* culture of human peripheral mononuclear cells with a commercial gene array (DUALChips® inflammation and breast cancer, Eppendorf, Germany). In the second step, potential target genes are validated in an *in vivo* experiment to prove the applicability of specific *in vivo* gene expression profiling in humans. Total RNA out of human whole blood samples were analyzed for specific gene expression profile of potential target genes as an indicator of the abuse of testosterone. Eight voluntary participants underwent controlled testosterone treatment and exercise intervention. In parallel, the effectiveness of the controlled testosterone application and physiological hormone levels were validated by two methods: indirectly by the urinary testosterone/epitestosterone (T/E) measurement as well as directly by salivary testosterone quantification.

Materials and methods

Study design

Nineteen male volunteers were separated into two subgroups. The first (n = 11; 38.6 ± 12.4 years; 82.8 ± 13.8 kg) served as a control group to demonstrate exercise induced gene expression effects. All participants of the control group had to absolve a standardized all-out incremental cycle ergometer test, up to subjective exhaustion. The ergometer tests were circadian matched to reduce time-dependent effects and was set in correspondence to the pharmacology of transdermal applied testosterone by gels, so that exercise commenced when levels of testosterone were significantly increased in blood plasma, correlating 5–6 h after application.[17] Blood collection both pre- and post-exercise was taken at 1 pm and 2 pm, respectively. The other 8 volunteers (38.1 ± 9.4 years; 85.4 ± 5.9 kg) had to pass a complex study design. To detect circadian rhythms in gene expression the sampling of whole blood, urine and saliva was collected at 8 am, 2 pm, and 5 pm. In a second step, the participants were to pass the same program and sampling by adding a cycle ergometry of 60 min at the individual anaerobic lactate threshold (IANS). After one week of rest, the participants of the second group passed the same two sampling regimes with the addition of adding a single dose of 1.5 mg testosterone per kilogram of body weight at 8 am on testing days. To evaluate long-term effects, all participants received two extra doses one and two weeks after the first application. Here, the sampling was carried out only in the evening at 5 pm. The hormone was administered transdermally through commercial testosterone gels (TestoGel®, Bayer, Germany). The daily testosterone dose was limited by the ethic committee to 1.5 mg kilogram of body weight which corresponds to the dose recommended by the manufacturer and correlating to hormone therapy for hypogonadism. To detect wash-out effects, all participants were to pass a further sampling one week after the last testosterone application. The study design was approved and accepted by the ethical committee of the medical faculty, Technische Universität München (project number: 1777/07).

Sample collection and preparation

At all sampling points, the collection of about 8 ml human whole blood was performed using four tubes of PAXGene® Blood RNA system (Quiagen/BD, GER) for total RNA extraction from human whole blood. All preparations were performed using the manufacture's guidelines.

The PAXGene® system enables direct stabilization of RNA within the process of sampling and as such storage effects on gene expression modification are minimized.[18] RNA quantity, quality, and purity were photometrically assessed using a Nanodrop (Peqlab, Germany). Only RNA samples were used where the optical dense ratio of 260/280 nm was higher than 1.8. To prevent cross-contamination with genomic DNA, a DNA digestion step was carried out which is supplied with the PAXGene® Blood RNA system. The mean yield of the total RNA extraction from 8 ml whole blood was located at 36.88 ± 7.72 µg of total RNA at a mean concentration of 115.24 ± 24.12 ng/µl and a mean 160/280 nm ratio of 2.11 ± 0.04. Additionally, to validate the RNA sampling procedure, selected samples were analyzed by capillary electrophoresis (Bioanalyzer 2100; Agilent Technology, Palo Alto, CA, USA) as described earlier.[12,18] The mean RNA integrity number (RIN) of selected samples was 8.4 ± 1.1. Urine and saliva were collected by using sterile urine cups and SaliTubes (Sarstedt, Germany), respectively. With saliva sampling, all participants used sterile straws to salivate directly into the SaliTubes preventing cross-contamination of the samples by hormonal residues on the hands. Total RNA, urine and saliva were stored at −80 °C until analysis.

Hormone analysis

To validate the effectiveness of the transdermal hormone application, two different methods were used: (1) the classical testosterone/epitestosterone (T/E) quotient in urine and (2) the total salivary testosterone concentration. T/E-quotients of urine

samples were analyzed in an accredited anti doping laboratory (Institute of Doping Analysis and Sports Biochemistry, Kreischa, Germany) using the standard MS method described by Grosse *et al.*[19] Salivary testosterone concentration was measured by a commercial enzyme linked immunoassay (Salivary Testosterone ELISA SLV-3013; DRG Diagnostics, Marburg, Germany) following manufacturer guidelines using SUNRISE plate reader (Tecan, Crailsheim, Germany).

Selection of potential target genes and primer validation

To explore a potential target gene of testosterone application, human peripheral blood mononuclear cell cultures from one male volunteer were treated with 0.1 μM testosterone for different time spans (6, 12, and 24 h). In this preliminary experiment, the testosterone-treated samples were analyzed and compared to untreated samples using two different sets of commercially available DUALChips® (inflammation and breast cancer, Eppendorf, Germany) to increase the number of the tested genes. Potential testosterone sensitive genes, which were identified and regulated by Sivlerquant® Detection software (Eppendorf, Hamburg, Germany), were selected to be tested *in vivo*. All array data were generated by using Silverquant® Scanner (Eppendorf, Hamburg, Germany).

The primers for the selected genes 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 performed using primer design program primer 3 (http://frodo.wi.mit.edu/) in respect to the following factors of optimization: (1) primer dimer formation, (2) self-priming of the primer pair, and (3) primer annealing temperature at 60 °C. Where possible, primer pairs were designed exon-spanning to discriminate false positive PCR results. Designed primers were ordered, synthesized and shipped by MWG Biotech (Ebersberg, Germany). Primer testing was performed with human reference RNA (Stratagene® QPCR Human Reference Total RNA, Stratagene, La Jolla, USA). The generated PCR products were checked for DNA amplification, product length and primer dimer formation by agarose gel electrophoresis. All primer sequences, their amplicon size, annealing and detection temperatures for quantitative real-time PCR are illustrated in Table 1.

Gene amplification and measurement

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, CA, USA) using the standard protocol of the manufacturer. For all RNA quantifications a standard 10 μl real-time RT-PCR mix was used: 5.0 μL 2x Quanti Tect SYBR Green-Mix, 0.4 μl for each primer (20 μM), 0.1 μl Quanti Tect RT mix, 3.1 μl RNAase free water and 1.0 μl of total RNA (10 ng/μl). All one-step RT-PCR runs were performed using the following quantification protocol: reverse transcription at 50 °C for 30 min, initial PCR activation at 95 °C for 15 min, followed by 40 PCR cycles of annealing at primer specific temperature (Table 1) for 30 s, primer extension at 72 °C for 30 s, fluorescence detection at PCR-product specific temperature (Table 1), and at least denaturation at 94 °C for 15 s. All PCR quantifications were closed by performing a melting curve analysis. Threshold cycle (Ct) and melting curves were acquired using the quantitation and melting curve program Rotor-Gene 6000 Series Software (v1.7). 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 1were baseline corrected and the threshold was set manually (using identical threshold levels for one gene in all analyzed samples). Gene expression data were processed applying relative quantification method on the basis of the ΔΔCt-method.[20] In the initial step, all Ct levels were set according to a calibrator sample to adjust inter-run variation. In the second step, the gene expression changes were corrected by using internal housekeeping genes indicating the lowest variation over all samples. For this purpose, several potential housekeeping genes, known from literature and DUALChip®, were tested for applicability in the normalization process: HPRT1, YAWHAZ, GAPDH from whole blood cells,[18] PPIP from peripheral blood cells,[21] ubiquitin from hepatocellular carzinoma,[22] histone 3 from white blood cell culture,[23] and ACTB, HK1, and MDH1 as reference genes which were spotted as housekeeping genes amongst others on the DUALChip®. For the normalization of target gene expression the arithmetic mean of the validated reference genes were taken, as previously reported by Reiter *et al.*[12]

Data analysis and statistics

Gene expression data quantified by real-time PCR over the factors of exercise, treatment, time and participant were calculated by using a multiple ANOVA. All statistical analysis was performed using the automated statistical software tool SigmaPlot 10 Software (Systat Inc., Erkrath, Germany) and a p-value of 0.05. In a first step, the Kolmogorov-Smirnov test (with Lilliefors' correction) was used to test data for normality. If normality failed, an ANOVA on ranks was performed using a Tukey-test which is less conservative than the Bonferroni t-test. Correlation analysis of interval scaled data was performed using the Pearson Product Moment Correlation. All data are illustrated by means ± standard deviation (SD).

Results

Hormone quantification

To prove the effectiveness of the transdermal testosterone application, standardized methods were used to show a total increase of whole body testosterone concentration. Figure 1 illustrates the measured T/E quotient in the urine samples of all participants. The mean value over all participants is illustrated by the solid line. All basal levels of T/E without additional testosterone range 1.0 ± 0.4, including the sample before the first testosterone application day (T 8 am). In context of circadian rhythm no variation of T/E could be detected during the control days at the time points of control 8 am, 2 pm, and 5 pm. The endurance exercise test did not alter the T/E quotient directly after Control Ex 2 pm nor 3 h after the exercise (Control Ex 5 pm). After 6 h of transdermal testosterone application (T 2 pm) an apparent increase of T/E was visible reaching significant levels of 2.4 ± 1.2 after 9 h (T 5 pm). Combining the testosterone application and endurance exercise resulted in a dramatic increase of T/E quotient (up to 4.0 ± 3.3) 3 h post exercise (T Ex 5 pm). The dramatic increase following exercise and testosterone administration was attributed by two participants. The two individual T/E profiles of the corresponding participants P1 (dotted line) and P8 (dashed line) are illustrated in Figure 1. In this case, the two volunteers achieved T/E levels up to 9.3. The other participants exhibited levels between 1.3 up to 4.0. After one week of wash-out, the urinary T/E quotients reached basal level of 1.0 ± 0.6 in all participants.

Table 1. Primer specifications

Gene	Sequence forward primer 5′-3′	Sequence Reverse Primer 5′-3′	Product length [bp]	AT	DT	Gene ID
AR	GGGCACTTCGACCATTTCT	GTTCTCCAGCTTGATGCGA	493	55 °C	82,5 °C	NM_000044.2
BCL2	CACCTGTGGTCCACCTGAC	TACAGTTCCACAAAGGCATCC	337	62 °C	85 °C	NM_000633.2
ACTB	CCAAGGCCAACCGTGAGAAGAT	CCACGTTCCGTGAGGATCTTCA	255	62 °C	82 °C	NM_001100.3
CAV1	CGTAGACTCGGAGGGACATC	CAGGAAGCTCTTAATGCATGG	397	60 °C	80,5 °C	NM_001753.4
CCL8	GACTTGCTCAGCCAGATTCA	ATCCCTGACCCATCTCTCCT	195	58 °C	76,0 °C	NM_005623
PPIB	TACAAAAGTGAGTCCATGGGC	ATCGCCAAGGAGTAGGGC	163	62 °C	83 °C	NM_000942.4
FGF8	AGCTGATCGCCAAGAGCA	GGCGGGTAGTTGAGGAACTC	250	65 °C	83 °C	NM_033165.3
GAPDH	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC	189	62 °C	82 °C	NM_002046.3
H3	ACTCGCTACAAAAGCCGCTC	ACTTGCCTCCTGCAAAGCAC	232	62 °C	80 °C	NM_002107.3
HK1	AGACGCACCCACAGTATTCC	AAGATCCAGGGCCAAGAAGT	347	62 °C	84 °C	M75126.1
HPRT1	ACGTCTTGCTCGAGATGTGA	CTTGCGACCTTGACCATCTT	345	62 °C	76 °C	NM_000194.2
IGFBP3	TGCTAGTGAGTCGGAGGAAGA	CTACGGCAGGGACCATATTC	237	58 °C	80 °C	NM_000598.4
IL1beta	CGATGCACCTGTACGATCAC	TTCTGCTTGAGAGGTGCTAG	388	62 °C	80 °C	NM_000576.2
IL6	TACCCCCAGGAGAAGATTCC	TTTCAGCCATCTTTGGAAGG	204	62 °C	78 °C	NM_000600.2
MDH1	CATTCTTGTGGGCTCCATG	TGAGTCGAGGAATGGTTTCC	321	62 °C	78 °C	NM_005917.2
PCNA	GTCCAAAATACTAAAATGCGCC	TCACTCCGTCTTTTGCACAG	278	62 °C	77 °C	NM_002592.2
PTGS2	CCCTTGGGTGTCAAAGGTAA	GGCACCAGACCAAAGACCT	404	55 °C	78,5 °C	NM_000963.2
SRA1	ATGGATCCCCCAGAGTCC	GGTGGCTTGAAAGCTCTTG	342	65 °C	83 °C	NM_001035235.2
UBI	AGATCCAGGATAAGGAAGGCAT	GCTCCACCTCCAGGGTGAT	198	62 °C	84 °C	NM_018955.2
VEGFa	CCACCATGCCAAGTGGTC	GAGGTTTGATCCGCATAATCTG	281	63 °C	81,5 °C	AF022375.1
VEGFb	GCCACCAGAGGAAAGTGGT	CCTGTCTGGCTTCACAGCA	323	60 °C	85,5 °C	NM_003377.3
VEGFc	CGGATGCTGGAGATGACTC	TCGTACATGGCCGTCTGTAA	372	60 °C	78,5 °C	NM_005429.2
YWHAZ	TCATCTTGGAGGGTCGTCTC	AGTTAAGGGCCAGACCCAGT	352	62 °C	77 °C	M86400.1

AT = annealing temperature; DT = detection temperature, bp = base pairs

AR = androgen receptor; BCL2 = B-cell CLL/lymphoma 2; ACTB = actin beta; CAV1 = caveolin 1; CCL8 = chemokine (C-C motif) ligand 8; PPIB = peptidylprolyl isomerase B (cyclophilin B); FGF8 = fibroblast growth factor 8; GAPDH = glycerin aldehyde dehydrogenase; H3 = histone 3; HK1 = hexokinase 1; HPRT1 = hypoxanthine phosphoribosyltransferase 1; IGFBP3 = insulin-like growth factor binding protein 3; IL1ß = interleukin-1 beta; IL6 = interleuklin-6; MDH1 = malate dehydrogenase-1; PCNA = proliferating cell nuclear antigen; PTGS2 = prostaglandin-endoperoxide synthase 2; SRA1 = steroid receptor RNA activator protein; UBI = ubiquitin; VEGFa/b/v = vascular endothelial growth factor A/B/C; YWHAZ = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

In parallel to the urinary T/E quotient, salivary testosterone concentrations were elucidated by ELISA (Figure 2). Likewise the T/E, salivary testosterone increased significantly within several hours from basal levels to between 49.5 pg/ml and 4394.5 pg/ml. It has to be mentioned that in some cases, the salivary testosterone concentrations exceeded as the highest ELISA standard value of 5000 pg/ml. If so, the concentration of the sample was set to 5000 pg/ml. Salivary testosterone levels stayed significantly high during supplementation phase until 3 h post exercise. In this case, the levels dropped to 2575.2 pg/ml. Additionally, in spite of the one-week wash-out period, testosterone levels were still above basal levels in salivary samples. The direct comparison of urinary T/E and salivary testosterone level by the

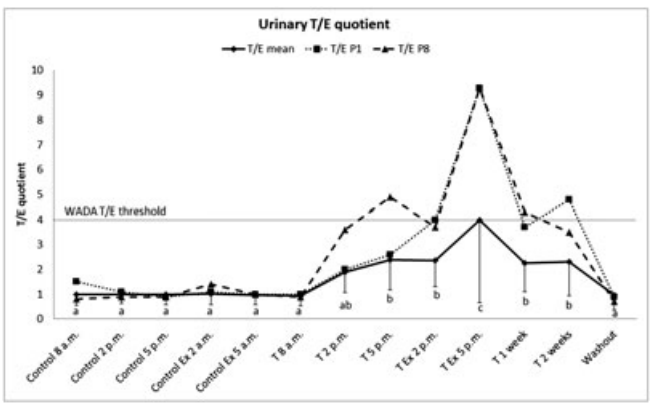

Figure 1. Mean values + − SD of T/E quotient in urinary samples (solid line); P1 = proband 1 (dotted line); P8 = proband 8 (dashed line). Ex = exercise. T = testosterone application (1.5 mg per kilogram body weight). Significant difference ($p < 0.05$) is indicated by letters.

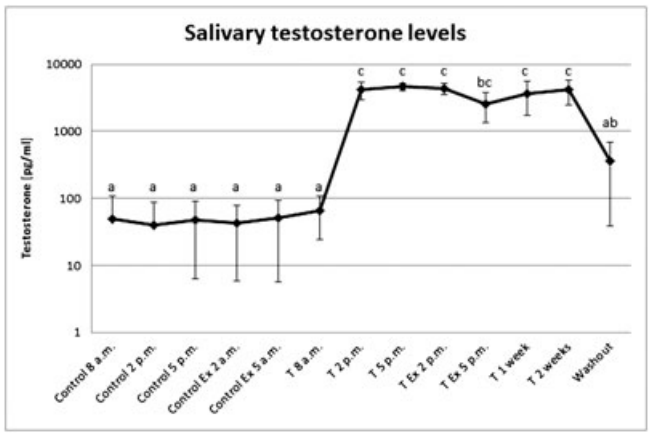

Figure 2. Mean values + − SD of testosterone levels in salivary samples. Data are depicted logarithmized. Ex = exercise. T = testosterone application (1.5 mg per kilogram body weight). Letters indicates significant difference ($p < 0.05$).

Pearson Product Moment Correlation indicated a highly significant correlation ($p < 0.001$) with a moderate correlation coefficient of 0.425. In this case, the two participants P1 and P8 with high urinary T/E quotients were in range of the group.

Gene expression

In the evaluation of a suitable housekeeping gene for the normalization process, a set of nine classical reference genes were tested for significant variation within the groups of exercise, hormone intervention and time. In this context, the focus was set on the two interventions: only exercise in an all-out test protocol and endurance exercise (60 min at the IANS) in combination with/without additional testosterone and time. The statistical analysis indicates that only one gene, PPIB (cyclophilin b), is stable over all tested variables (Table 2). All other genes were more or less under a significant influence on the mentioned factors. HPRT1, GAPDH, UBI, and MDH1 were unregulated in context of all-out exercise. Whereby, YWHAZ, ACTB, and HK1 were stable against the influence of endurance exercise, time, and testosterone. To avoid the risk of modulating the gene expression data by a normalization process with significant regulated housekeeping genes, the Ct values of potential target gene were normalized only to one housekeeping gene, in this case to PPIP.

For the determination of potential biomarkers indicating testosterone application, several genes were tested demonstrating possible gene expression modulation under the influence of testosterone in blood cells. A set of 14 genes, validated in a previous in vitro cell culture study [13] by gene array analysis, were used to test the hypothesis. All genes were normalized to the internal reference gene PPIB (see results above). Table 3 gives an overview about the target genes, which demonstrated significant group effects on exercise, testosterone or time. In sum, five genes (AR, BCL2, PCNA, IGFBP3 and VEGFb) out of 14 target genes were not significantly regulated by exercise nor testosterone or time. Here, AR expression levels tended to decrease in the case of long-term testosterone application but did not reach significant levels (Figure 3). After a three-week long-term supplementation (day 22) AR was reduced to the 0.7-fold in comparison to experimental onset. Four genes (IL1beta, PTGS2, VEGFa, and CAV1) showed dependency to almost all group factors in this case.

In the testosterone intervention group, eight genes showed a distinct dependency on one or more factors. Table 4 lists the different kinds of modulations, which could be elucidated for each target gene. The results indicate that almost all genes (LI1ß, CCL8, PTGS2; VEGFa; CAV1, SRA, and FGF8) are dependent on one or more of the following factors: circadian rhythm, acute endurance exercise or testosterone treatment. With one exception, IL6 seemed to be stable over both time of day and exercise but was significantly reduced after the third testosterone application in the long-term experiment. Long-term application led to a 0.4-fold reduction, which is indicated by increased Ct-values in the real-time PCR (Figure 4).

One further reason for the high variation in gene expression results is the possibility of an individual variation in expression height of the tested genes. Therefore, to show an overall expression level within each proband the mean Ct value of all measured target genes of each proband were compared to each other (Table 5). All 14 target genes indicated a significant influence of the individual in expression height. The highest individual range between the lowest and highest individual mean Ct value was measured for IL-6 (3.719 Ct), followed by IGFBP3 (2.390 Ct) and VEGF-c (2.102 Ct).

Table 2. Significant group effects ($p < 0.05$) on housekeeping genes in matters of exercise and/or testosterone treatment and time in whole blood cells

Reference Gen	Control group (n = 11) (prae vs. post exercise)	Intervention group (n = 8) (exercise/testosterone/time)
HPRT1	-	significant changes
GAPDH	-	significant changes
YWHAZ	significant changes	-
PPIB	-	-
ACTB	significant changes	-
H3	significant changes	significant changes
UBI	-	significant changes
HK1	significant changes	-
MDH-1	-	significant changes

ACTB = actin beta; GAPDH = glycerin aldehyde dehydrogenase; H3 = histone 3; HK1= ; HPRT1 = hypoxanthine phosphoribosyltransferase 1; MDH-1 = malate dehydrogenase-1; PPIB = peptidylprolyl isomerase B (cyclophilin B); UBI = ubiquitin; YWHAZ = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Discussion

The main focus in the present study was to evaluate a distinct gene expression pattern in whole blood samples which could indicate abuse of testosterone. Circadian rhythms and exercise were included into the study to show possible effects of these parameters in context of hormonal and gene expression changes. In addition, the authors validated the testosterone application by measuring urinary T/E quotient in combination with salivary testosterone concentration as an indicator of successful administration.

Hormone analysis

For testosterone application a transdermal procedure was used. To validate the existence of increased circulating testosterone in the blood, the concentration was measured indirectly by using salivary and urinary samples. Saliva reflects the bioavailable testosterone concentrations in the serum with a high correlation. [24–26] It can therefore be assumed that circulating blood cells are in contact with free testosterone in the serum. In the present study it could be shown that testosterone levels dramatically increase (up to an 80-fold increase) in the salivary samples within the first six up to nine hours after application. Comparing basal levels of salivary testosterone concentration with recent studies, measurement errors could be eliminated for the basal levels. Here, the concentrations reflect normal levels of free testosterone in the saliva. Additionally, it could be shown that at rest, the testosterone levels in the morning were slightly increased compared to those in the evening. This concurs with Ladman *et al.*[27] Under physiological conditions, salivary testosterone could increase over basal levels. For example, by short high-intensity exercise salivary levels raised about 35% over the basal concentration but did not reach such excess levels as measured here.[28,29] Therefore, the high concentration of testosterone has to be validated by other techniques to show accuracy of the ELISA test assay. It is possible that

Table 3. Significant group effects ($p < 0.05$) on target genes in matters of exercise and/or testosterone treatment and time in whole blood cells

Target Gen	Control group (n = 11) (prae vs. post exercise)	Intervention group (n = 8) (exercise/testosterone/time)	Involved gene function
AR	-	-	Hormon receptor
BCL2	-	-	Anti-apoptosis
CAV1	significant changes	significant changes	Plasma membrane/cell cycle
CCL8	-	significant changes	Inflammation
FGF8	-	significant changes	Growth factor/connective tissue
IGFBP3	-	-	Inflammation
IL1β	significant changes	significant changes	Inflammation
IL6	-	significant changes	Inflammation
PCNA	-	-	Cell cycle/DNA synthesis
PTGS2	significant changes	significant changes	Prostaglandin biosynthesis/inflammation
SRA	-	significant changes	Transcriptional coactivation
VEGFa	significant changes	significant changes	Growth factor/Vascularization
VEGFb	-	-	Growth factor/Vascularization
VEGFc	significant changes	-	Growth factor/Vascularization

AR = androgen receptor; BCL2 = B-cell CLL/lymphoma 2; CAV1 = caveolin 1; CCL8 = chemokine (C-C motif) ligand 8; FGF8 = fibroblast growth factor 8; IGFBP3 = insulin-like growth factor binding protein 3; IL1ß = interleukin-1 beta; IL6 = interleuklin-6; PCNA = proliferating cell nuclear antigen; PTGS2 = prostaglandin-endoperoxide synthase 2; SRA = steroid receptor RNA activator protein; VEGFa/b/v = vascular endothelial growth factor A/B/C

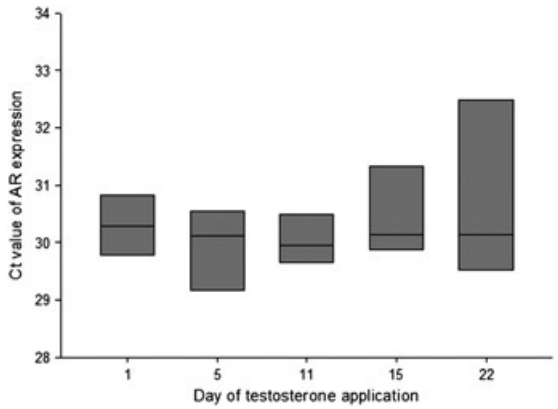

Figure 3. Cycle threshold (Ct) of androgen receptor (AR) gene expression in human whole blood cells. (1 = baseline. 5 = first testosterone application at day 5; 11 = second testosterone application at day 11; 15 = third testosterone application at day 15; 22 = after wash out at day 22).

commercial ELISAs are not validated for such high testosterone concentrations although some authors report salivary testosterone concentrations of about 4000 pg/ml after transdermal application in boys, which were measured by radio immunoassay.[30] Thus, the measured levels in the present study may be achievable. In addition, Kutsukake *et al.* have postulated for chimpanzees that salivary testosterone quantification could more sensitive to testosterone variation when compared to urinary quantification.[31] Furthermore, the authors had elucidated that salivary testosterone levels correlate to plasma levels but not to urinary levels.

Although a middle correlation between salivary testosterone and T/E quotient could have been shown here, the urinary T/E quotient did not increase to the extent of salivary testosterone. These diverging results may possibly be resultant of the pharmacokinetic properties of the testosterone gels. Both Geyer *et al.*[32] and Van Renterghem *et al.*[33] postulated only a slight increase of T/E quotient after transdermal application of almost equal amounts of testosterone by transdermal application. This may imply different steroid metabolism or diffusion processes in the salivary glands leading to different appearance of testosterone in saliva versus urine. Additionally, it has been shown that steroid hormones could pass the barrier between serum and saliva by passive and active processes dependent on their conjugated or unconjugated form.[29] Thus, testosterone, its metabolites and other steroid hormones possibly interfere with ELISA tests leading to increased concentration measurements. It is therefore necessary to prove both the salivary testosterone concentration and steroid profile by gas chromatography-mass spectrometry (GC-MS).

Gene expression

Both salivary and urinary hormone analyses indicated a significant increase of circulating testosterone in the blood stream. Consequently, the blood cells were in contact with increased testosterone levels. By the transdermal application of testosterone it has to be marked that testosterone liberation is creeping. One additional indicator of the increased hormone levels is the slight down-regulation of AR gene expression in the whole blood cells. But statistical analysis did not affirm a significant alteration. The AR repression as a consequence of increased serum testosterone levels is also reported for peripheral mononuclear blood cells in rats (Figure 5) as well as human muscle cells.[34] As such, a modulation of gene expression of potential target genes downstream the AR signaling pathway could be assumed. In PCR analytics it is common to normalize gene expression data against an almost unregulated internal reference gene. Consequently, in the present study nine conventional housekeeping genes were plotted against the factors: (1) circadian rhythms, (2) acute exercise, and (3) testosterone application. Only PPIB was elucidated to be independent of the influencing factors. Therefore, for the authors PPIP was the only suitable housekeeping gene for the normalization process. Using the housekeeping gene for semi-quantitative PCR, PPIB is also used by other authors for peripheral blood cells.[21] In contrast, Carrol *et al.*[18] reported that HPRT1, YWHAZ, and GAPDH are the three most stable transcripts using the same

Table 4. Significant variations of gene expression ($p < 0.05$) on target genes in matters of exercise and/or testosterone treatment and time in whole blood cells

Target Gene	Control vs. Exercise	Testo + Ex vs. Control + Ex	Testo + Ex vs. Testo - Ex	Testo vs. Control	Long term effect
IL1β	Ex ↑				
IL6					Testo ↓
CCL8	Ex ↑	circadian ↑ Testo ↓			
PTGS2	Ex + circadian ↑				
VEGFa	circadian ↑	Testo ↑	circadian ↑	Testo ↑	
CAV1		circadian ↓			
SRA	Ex + circadian ↑	circadian ↑		Testo ↑	
FGF8	circadian ↓ Ex ↑	circadian ↑ Testo ↓	circadian ↓		

Ex = exercise dependency; Testo = testosterone dependency; circadian = circadian dependency;
↑ = up-regulation of gene expression; ↓ = downregulation of gene expression
IL1ß = ilnerleukin-1 beta; IL6 = Interleuklin 6; CCL8 = chemokine (C-C motif) ligand 8; PTGS2 = prostaglandin-endoperoxide synthase 2; VEGFa = vascular endothelial growth factor A; CAV1 = caveolin 1; SRA = steroid receptor RNA activator protein

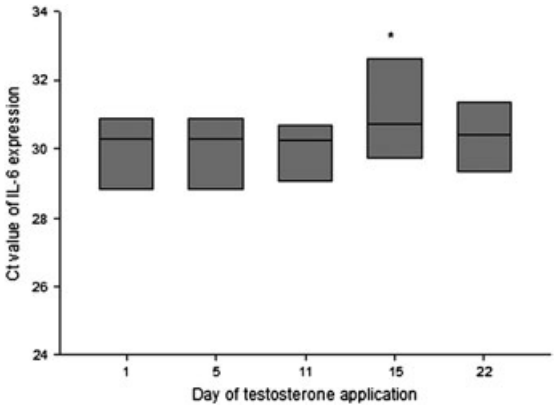

Figure 4. Cycle threshold (Ct) of interleukin 6 (IL6) gene expression in human whole blood cells. (1 = baseline. 5 = first testosterone application at day 5; 11 = second testosterone application at day 11; 15 = third testosterone application at day 15; 22 = after wash out at day 22).

techniques for blood collection and mRNA extraction as the present study; however, it has to be mentioned that Carrol *et al*. were working on pediatric patients and, as such, it can be assumed that the pubertal state of the individuals has a potential effect on the gene expression of potential unregulated genes. Additionally, Carrol *et al*. also tested the gene expression at one point in time. So, the difference between the studies could be explained by the different study design. Dheda *et al*.[35] are working on the validation of housekeeping genes in human bloods cells; they postulate that none of the common housekeeping genes are suitable as a calibrator for target gene expression. In addition, Dheda *et al*.[35] used peripheral blood mononuclear cells, a special cell fraction of the blood cultivated *in vitro*. These circumstances indicate that a direct comparison of the results *in vitro* versus *in vivo* has to be critically evaluated. This issue of *in vitro/in vivo* transformation is demonstrated by the results in present study as well. Here, potential testosterone sensitive genes were used which were elucidated by gene array analysis in an *in vitro* study. The results of the much more sensitive RT-PCR in the *in vivo* study revealed that only one gene IL-6 was exclusively dependent on testosterone application. In addition, the ad hoc supplementation of testosterone application in a cell culture could not be compared directly with a transdermal *in vivo* supplementation of testosterone. The pharmacological flow of testosterone into the blood stream is lingering and time dependent so the gradually increasing hormone levels could induce a different gene expression pattern. This assumption could be

Table 5. Significant variations of gene expression on target genes between different probands. Data are depicted as Ct-values (min = lowest Ct mean value; max = highest Ct mean value; SD = standard deviation; range = max minus min).

Proband	AR	BCL2	PCNA	IL1B-v2	IL-6	CCL-8	IGFBP3	PTGS2	VEGF-b	VEGF-c	CAV1	SRA	VEGF-a	FGF-8
P1	30.888	24.081	20.987	21.072	31.046	27.555	23.525	23.419	24.509	27.971	29.855	24.516	24.930	30.584
P2	30.232	23.782	20.325	20.429	30.026	26.887	22.745	22.363	23.318	29.110	29.657	24.072	25.080	30.577
P3	30.192	24.801	21.197	19.983	30.867	25.702	24.394	22.226	24.013	28.182	30.059	24.724	23.607	30.632
P4	30.683	24.499	21.493	20.826	30.113	27.826	23.507	22.843	24.049	28.693	29.918	24.963	24.072	31.684
P5	29.640	23.936	20.479	19.868	27.327	27.587	22.940	22.056	24.069	27.129	30.400	24.392	23.841	30.811
P6	30.440	24.638	21.590	20.642	30.921	27.637	24.964	22.833	24.249	29.231	31.022	25.824	24.192	31.501
P7	29.133	23.523	20.602	20.047	28.806	26.666	22.574	22.503	22.807	27.493	30.215	24.922	23.734	30.836
P8	30.519	24.478	21.660	21.130	29.907	27.590	24.200	23.653	23.734	27.907	29.242	25.501	24.117	32.220
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.016	<0.001	<0.001	<0.001
min	29.133	23.523	20.325	19.868	27.327	25.702	22.574	22.056	22.807	27.129	29.242	24.072	23.607	30.577
max	30.888	24.801	21.660	21.130	31.046	27.826	24.964	23.653	24.509	29.231	31.022	25.824	25.080	32.220
range	1.755	1.278	1.335	1.262	3.719	2.124	2.390	1.597	1.702	2.102	1.780	1.752	1.473	1.643
SD	0.5753	0.4527	0.5261	0.4968	1.2629	0.7206	0.8522	0.5662	0.5466	0.7481	0.5288	0.5771	0.5383	0.6174

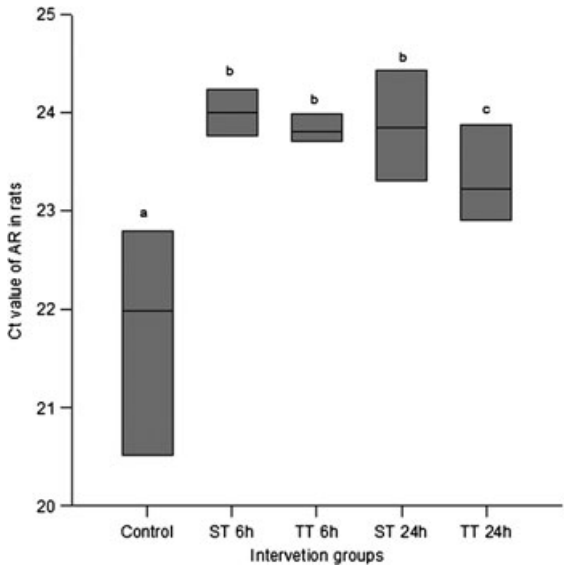

Figure 5. Cycle threshold (Ct) of androgen receptor (AR) gene expression in peripheral mononuclear blood cells (in rats) before and after in vivo testosterone application. (ST = sedentary + testosterone; TT = training + testosterone). Letters indicates significant difference ($p < 0.05$). Figure was adapted from Schönfelder *et al*.[13]

confirmed that many of the tested genes, housekeeping genes as well as potential target genes, are dependent on time and treatment. Furthermore, the direct comparison of the control group (all out exercise) with the intervention group (constant endurance exercise) indicates that exercise intensity must also be taken into account. In this case, PPIB was the most stable housekeeping gene. The dependency of gene expression on exercise intensity is possibly triggered by the activity of the autonomic nervous systems. Adrenaline and noradrenaline are strong modulators of the blood cell composition as well as blood cell function.[36] Consequently, quantification of gene expression by PCR in whole blood cells fraction is possibly affected by: (1) gene expression modulation by direct catecholamine influence; and (2) by a drift in differential blood cell count, so-called leukocytosis and lymphopenia.[37] This fact could also induce time dependent results because leukocytosis and lymphopenia are time-dependent events happing in the acute phase of exercise as well as in the post-exercise phase.

To complete the set of influencing factors, it has to be mentioned that all target genes diverged significantly in Ct values when data were compared between the individuals indicating individual expression levels (Table 5). This matter would be a critical point for data handling of gene expression results. High inter-individual differences decrease the statistical power.

To our knowledge, the present study is the first examination of cross-effect testing of the factors circadian rhythm, exercise and exogenous testosterone application with semi-quantitative PCR analytics. In addition to the nine tested housekeeping gene, 14 possible target genes of testosterone treatment were quantified in whole blood cell mRNA extracts. The present results indicate that all tested factors could more or less interfere with the expression of the elucidated genes in whole blood cell extracts.

Only IL6 was elucidated as the single possible biomarker for testosterone treatment. IL6 mRNA expression was affected neither by exercise nor by circadian rhythm in the present study. Only a repeated application of testosterone led to a down-regulation of IL6 mRNA expression in whole blood cells. In comparison with recent results in cultivated human macrophages from older men and postmenopausal women testosterone did not affect the expression of IL6.[38] In this context Kim *et al*. postulated that IL6 production might be interactively influenced by age and menopause.[39] Here, the estrogen deprivation after menopause may enhance blood cell cytokine production in postmenopausal women. Additionally, results from prostate cancer studies indicate that IL6 increase testosterone production in LNCaP cells.[40] Thus in reverse, increased serum testosterone levels possibly could repress IL6 expression by a hypothetical negative feedback mechanism. Taken together, although IL6 has shown a significant dependency on testosterone the conflicting results from recent literature and the possible age and estrogen effects lead to the assumption that IL6 could not be seen as an exclusive indicator for testosterone application.

From a physiological point of view, the tested target genes could be functionallyclustered (Table 3). This analysis indicates that most of the target genes could be grouped into two main functional groups: inflammation processes (CCL8, IGFBP3, IL1β, IL6, PTGS2) and growth factors (FGF8, VEGFa, VEGFb, VEGFc). Both groups are known to be highly influenced by acute as well as chronic exercise.

In conclusion, the present study indicates that only one target gene has shown a possible dependency on elevated testosterone levels in whole blood cells. All other PCR analytics shown here represent an indirect method, underlying high biological variations. Figure 6 sums up the critical factors, which have to be taken into account if the gene expression technique could be used in doping analysis. The present study indicates that the biological system of sportsmen is highly variable. The orchestra of critical factors, such as individuality, exercise regime, pharmacology of application, choice of tissue and methods of analysis may indicate that it is necessary to develop individual gene expression profiles in parallel to individual hormone profiles. At the moment it is much more challenging to determine indirect biological markers indicating testosterone application as to detect directly for banned substances therefore more genes have to be included to find a specific gene cluster which indicates distinct misuse of testosterone. Beyond that, testosterone concentration underlies biological variation, which also could affect gene expression pattern in a natural way. Potentially, gene array analytics could explore more possible candidate genes clarifying the purpose. The present study indicates that the high variation between the individuals could be based on individual metabolic processes on steroid hormones therefore, the time factor and bioavailability

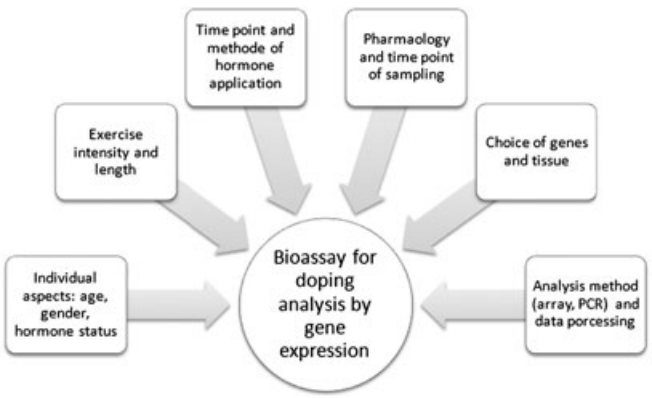

Figure 6. Summary of critical factors influencing which has to be followed for gene expression based bioassays in doping analysis.

could be a critical factor, indicating a high standardized testing regime in doping analysis and the analysis of hormone quantities. Furthermore, the present study has shown that salivary testosterone quantification could be an additional research method for doping analytics. Recent studies including this one indicate that salivary hormone concentrations correlate to urinary T/E quotient which could open a new possibility to detect the misuse of anabolic steroids by an oral and non-invasive method.

Acknowledgements

The authors acknowledge financial support from Bundesinstitut für Sportwissenschaft and the World Anti-Doping Agency.

References

[1] F. Hartgens, H. Kuipers. Effects of androgenic-anabolic steroids in athletes. *Sports Med.* **2004**, *34*, 513.
[2] R. Singh, S. Bhasin, M. Braga, J. N. Artaza, S. Pervin, W. E. Taylor, V. Krishnan, S. K. Sinha, T. B. Rajavashisth, R. Jasuja. Regulation of myogenic differentiation by androgens: Cross talk between androgen receptor/beta-catenin and follistatin/transforming growth factor-beta signaling pathways. *Endocrinology* **2009**, *150*, 1259.
[3] L. H. Duntas, C. Parisis. Doping: a challenge to the endocrinologist. A reappraisal in view of the Olympic Games of 2004. *Hormones* **2003**, *2*, 35.
[4] R. C. Hickson, S. M. Czerwinski, M. T. Falduto, A. P. Young. Glucocorticoid antagonism by exercise and androgenic-anabolic steroids. *Med. Sci. Sports Exerc.* **1990**, *22*, 331.
[5] R. C. Griggs, W. Kingston, R. F. Jozefowicz, B. E. Herr, G. Forbes, D. Halliday. Effect of testosterone on muscle and muscle protein synthesis. *J. Appl. Physiol.* **1989**, *66*, 498.
[6] C. M. Kuhn. Anabolic Steroids. *Recent Prog. Horm. Res.* **2002**, *57*, 411.
[7] D. Clasing, R. K. Müller. *Dopingkontrolle 2006*, 4th ed. Bundesinstitut für Sportwissenschaften: Bonn, Germany **2006**, 25.
[8] W. Schänzer. Metabolism of anabolic androgenic steroids. *Clin. Chem.* **1996**, *42*, 7.
[9] D. Thieme, J. Grosse, H. Sachs, R. K. Mueller. Analytical strategy for detecting doping agents in hair. *Forensic Sci. Int.* **2000**, *107*, 335.
[10] P. Anielski, D. Thieme, A. Schlupp, J. Grosse, F. Ellendorf, R. K. Mueller. Detection of testosterone, nandrolone and precursors in horse hair. *Anal. Bioanal. Chem.* **2005**, *383*, 903.
[11] M. Reiter, V. M. Walf, A. Christians, M. W. Pfaffl, H. H. D. Meyer. Modification of mRNA expression after treatment with anabolic agents and its usefulness for gene expression-biomarkers. *Anal. Chim. Acta* **2006**, *586*, 73.
[12] M. Reiter, M. W. Pfaffl, M. Schönfelder, H. H. D. Meyer. Gene expression in hair follicle dermal papilla cells after treatment with stanozolol. *Biomark Insights.* **2008**, *23*, 1.
[13] M. Schönfelder, M. Reiter, M. W. Pfaffl, H. H. D. Meyer, D. Kwiatkowska, R. K. Müller, R. Oberhoffer. Genexpressionsscreening als unterstützende Methode in der klassischen Doping-Analytik. *Deutsche Zeitschrift für Sportmedizin*, **2009**, *60*, 196.
[14] O. N. Akram, C. Bursill, R. Desai, A. K. Heather, R. Kazlauskas, D. J. Handelsman, G. Lamber. Evaluation of androgenic activity of nutraceutical-derived steroids using mammalian and yeast in vitro androgen bioassays. *Anal. Chem.* **2011**, *83*, 2065.
[15] P. Diel, A. Friedel, H. Geyer, M. Kamber, U. Laudenbach-Leschowsky, W. Schänzer, M. Thevis, G. Vollmer, O. Zierau. Characterisation of the pharmacological profile of desoxymethyltestosterone (Madol), a steroid misused for doping. *Toxicol. Lett.* **2007**, *169*, 64.
[16] A. Paparini, F. Impagnatiello, A. Pistilli, M. Rinaldi, G. Gianfranceschi, E. Signori, A. M. Stabile, V. Fazio, M. Rende, V. Romano Spica. Identification of candidate genes and expression profiles, as doping biomarkers. *Ann. Ig.* **2007**, *19*, 303.
[17] K. M. Lakshman, S. Basaria. Safety and efficacy of testosterone gel in the treatment of male hypogonadism. *Clin. Interv. Aging* **2009**, *4*, 397.
[18] E. D. Carrol, F. Salway, S. D. Pepper, E. Saunders, L. A. Mankhambo, W. E. Ollier, C. A. Hart, P. Day. Successful downstream application of the paxgene blood RNA system from small blood samples in paediatric patients for quantitative PCR analysis. *BMC Immunol.* **2007**, *12*, 8.
[19] J. Grosse, P. Anielski, H. Sachs, D. Thieme. Ethylglucuronide as a potential marker for alcohol-induced elevation of urinary testosterone/epitestosterone ratios. *Drug Test. Analysis* **2009**, *1*, 526.
[20] K. J. Livak, T. D. Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. *Methods* **2001**, *25*, 402.
[21] A. Pachot, J. L. Blond, B. Mougin, P. Miossec. Peptidylpropyl isomerase B (PPIB): a suitable reference gene for mRNA quantification in peripheral whole blood. *J. Biotechnol.*, **2004**, *114*, 121.
[22] V. R. Cicinnati, Q. Shen, G. C. Sotiropoulos, A. Radtke, G. Gerken, S. Beckebaum. Validation of putative reference genes for gene expression studies in human hepatocellular carcinoma using real-time quantitative RT-PCR. *BMC Cancer* **2008**, *8*, 350.
[23] J. Sehm, J. Polster, M. W. Pfaffl. Effects of varied EGCG and (+)-catechinconcentrations on proinflammatory cytokines mrna expression in cona-stimulated primary white blood cell cultures. *J. Agric. Food Chem.* **2005**, *53*, 6907.
[24] E. Sannikka, P. Terho, J. Suominen. Testosterone concentrations in human seminal plasma and saliva and its correlation with non-protein-bound and total testosterone levels in serum. *Int. J. Androl.* **2008**, *6*, 319.
[25] J. E. Morley, I. H. M. Perry, P. Patrick. Validation of salivary testosterone as a screening test for male hypogonadism. *Aging Male.* **2006**, *9*, 165.
[26] E. Papacosta, G. P. Nassis. Saliva as a tool for monitoring steroid, peptide and immune markers in sport and exercise science. *J. Sci. Med. Sport.* **2011** Sep; *14*(5), 424–34.
[27] A. D. Landman, L. M. Sanford, B. E. Howland. Testosterone in human saliva. *Experientia* **1976**, *32*, 940.
[28] B. T. Crewther, T. E. Lowe, J. Ingram, R. P. Weatherby. Validating the salivary testosterone and cortisol concentration measures in response to short high-intensity exercise. *J. Sports Med. Phys. Fitness* **2010**, *50*, 85.
[29] R. Gatti, E. F. De Palo. An update: salivary hormones and physical exercise. *Scand. J. Med. Sci. Sports* **2011**. *21*, 157.
[30] A. Mayo, H. Macintyre, A. M. Wallace, S. F. Ahmed. Transdermal testosterone application: pharmacokinetics and effects on pubertal status, short-term growth, and bone turnover. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 681.
[31] N. Kutsukake, K. Ikeda, S. Honma, M. Teramoto, Y. Mori, I. Hayasaka, R. Yamamoto, T. Ishida, Y. Yoshikawa, T. Hasegawa. Validation of salivary cortisol and testosterone assays in chimpanzees by liquid chromatography-tandem mass spectrometry. *Am. J. Primatol.* **2009**, *71*, 696.
[32] H. Geyer, U. Flenker, U. Mareck, P. Platen, T. Piper, A. Schmechel. The detection of the misuse of testosterone gel, in *Proceedings of the 25th Manfred Donike workshop*, *Vol. 15*, (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck). Sport & Buch Strauß: Cologne, **2007**.
[33] P. Van Renterghem, P. Van Eenoo, F. T. Delbeke. Population based evaluation of a multi-parametric steroid profiling on administered endogenous steroids in single low dose. *Steroids* **2010**, *75*, 1047.
[34] M. D. Roberts, V. J. Dalbo, S. E. Hassell, C. M. Kerksick. The expression of androgen-regulated genes before and after a resistance exercise bout in younger and older men. *J. Strength Cond. Res.* **2009**, *23*, 1060.
[35] K. Dheda, J. F. Huggett, S. A. Bustin, M. A. Johnson, G. Rook, A. Zumla. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques* **2004**, *37*, 112.
[36] R. J. Benschop, M. Rodriguez-Feuerhahn, M. Schedlowski. Catecholamine-induced leukocytosis: early observations, current research, and future directions. *Brain Behav. Immun.* **1996**, *10*, 77.
[37] J. B. Hansen, L. Wilsgård, B. Osterud. Biphasic changes in leukocytes induced by strenuous exercise. *Eur. J. Appl. Physiol. Occup. Physiol.* **1991**, *62*, 157.
[38] M. P. Corcoran, M. Meydani, A. H. Lichtenstein, E. J. Schaefer, A. Dillard, S. Lamon-Fava. Sex hormone modulation of proinflammatory cytokine and C-reactive protein expression in macrophages from older men and postmenopausal women. *J. Endocrinol.* **2010**, *206*, 217.
[39] O. Y. Kim, J. S. Chae, J. K. Paik, H. S. Seo, Y. Jang, J. M. Cavaillon, J. H. Lee. Effects of aging and menopause on serum interleukin-6 levels and peripheral blood mononuclear cell cytokine production in healthy nonobese women. *Age (Dordr)*. **2011**. DOI: 10.1007/s11357-011-9244-2
[40] J. Y. Chun, N. Nadiminty, S. Dutt, W. Lou, J. C. Yang, H. J. Kung, C. P. Evans, A. C. Gao. Interleukin-6 regulates androgen synthesis in prostate cancer cells. *Clin. Cancer Res.* **2009**, *15*, 4815.