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The analysis of the transcriptome as a new approach for biomarker development to trace the abuse of anabolic steroid hormones

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The abuse of anabolic steroid hormones in human sports and animal husbandry is an ubiquitous problem and therefore a tight control program in both areas is very important. Within these control programs, hormone residues are detected by immunoassays or chromatographical methods in combination with mass spectrometry. With these methods, all known substances can be detected; yet new xenobiotic growth promoters and new ways of application are difficult to detect. Therefore it is important to develop new sensitive screening methods to enable an efficient control for misused anabolic substances. The detection of their physiological action is a promising approach. Anabolic steroid hormones directly influence the expression of specific genes and thus the analysis of the transcriptome of different target tissues and matrices is of great interest. This review describes our recent efforts made concerning the analysis of gene expression changes in different tissues, different species and under different anabolic treatments.

Keywords: anabolic steroids; gene expression biomarkers; RT-qPCR

Introduction

Steroid hormones, like testosterone and estradiol, are involved in endocrine and paracrine regulation of growth in different tissues. In vertebrates, the number of skeletal muscle fibres is fixed at birth. Postnatal muscle growth results from hypertrophy of existing muscle fibres. During muscle hypertrophy, mononucleated satellite cells located on the periphery of the muscle fibres fuse with the muscle fibre cells providing the nuclei needed for an increased production of muscle proteins.[1–3] Insulin-like growth factor 1 (IGF-1) and its binding proteins (IGFBP) play a crucial role in muscle growth, for example by increasing the number of satellite cells and so enhancing muscle hypertrophy.[1] Treatment with anabolic steroid hormones increases the production of IGF-1 in the liver and also in skeletal muscle tissue.[4] This results in an increased growth rate and so IGF-1 is a mediator of the growth-promoting effect of steroid hormones in muscle.

Bone remodelling is a process that includes different kinds of cells and proteins. Osteoclasts, secret proteolytic enzymes which are responsible for bone tissue loss. Osteoblasts differ from osteocytes, which in combination with different minerals, form the bone matrix. Steroid hormones like estrogens and also androgens inhibit bone breakdown by stimulation of proliferation and differentiation of osteoblasts or by stimulating the generation of extracellular bone matrix proteins like Type I collagen, osteocalcin, or osteonektin. Anabolic steroid hormones show pro-apoptotic effects on osteoclasts and anti-apoptotic effects on osteoblasts and osteocytes. Hence they positively affect bone density.[5] The decrease of the endogenous production of anabolic sex hormones after menopause has a negative effect on bone mineral density leading to osteoporosis.[6]

Steroid hormones, mainly estrogens, show direct effects on adipose tissue by down-regulating lipogenesis and up-regulating lipolysis and the β-oxidation. Estrogens also decrease the differentiation rate of adipocyte-precursor cells to mature adipocytes.[7–9]

These positive effects on muscle mass and bone density and the mobilization of adipose tissue by anabolic steroid hormones are responsible for their misuse as growth promoters in sports and animal husbandry. To uncover this abuse, hormone residues are routinely detected using immunoassays or chromatographical methods in combination with mass spectrometry.[10–13] With these methods, only known substances can be discovered but new xenobiotic anabolic agents or hormone cocktails can hardly be detected. Therefore the development of new, sensitive detection methods is a permanent challenge for scientists.

In molecular medicine – for example, in cancer research – the development of molecular biomarkers is already a common approach for diagnostic purpose. Plasma biomarkers are developed for prognostic use and tumour biomarkers are applied to develop treatment strategies for each individual patient.[14,15] A promising way for the development of such biomarkers for the detection of the abuse of anabolic steroids is the analysis of physiological changes caused by treatment using '-omic technologies' like transcriptomics, proteomics, and metabolomics.[15–18] The choice of the right -omic technology for the specific application is a big challenge.

Steroid hormone receptors belong to the family of nuclear receptors and show a high affinity to their corresponding hormone.[19,20] After binding of the hormone or active analoga, the hormone-receptor complex directly influences the transcription of

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specific genes either by binding to specific DNA sequences in the promoter region of the genes or by activating other transcription factors which then activate the expression of specific genes.[18] Due to this direct effect on gene transcription, the analysis of changes in the transcriptome is a promising way for detecting physiological changes caused by treatment with anabolic steroid hormones.

This review reflects our in-house data obtained from different studies regarding the effects of anabolic steroid hormones on the transcriptome in different tissues and species. In all studies, analyzed target genes were chosen by screening the actual literature for steroidal effects on the physiology of the specific organs and their expression was quantified using RT-qPCR.

Tissue distribution of steroid hormone receptors

The first step in establishing physiological biomarkers is the choice of the ideal tissue. Therefore physiological knowledge about effects of steroid hormones in different tissues is needed. The precondition of direct steroidal action on a tissue is the presence of the specific receptors especially of the androgen receptor (AR) and the two isoforms of the estrogen receptor (ERα and ERβ). The primary group of tissues that are directly influenced by sex steroids are parts of the reproductive tract. In females, uterus, ovary, mammary gland, and vaginal epithelial cells will be of interest. All these organs express all three types of steroid receptors whereas in uterus, mammary gland and vaginal epithelial cells ERα and in ovary ERβ is the prevalent isoform of the estrogen receptor.[21–26] In the male organism, testis and prostate will be of major interest. In both tissues the androgen receptor expression is very high and estrogens mainly act via ERβ.[21,22,27–29] Other tissues that seem to be interesting are different kinds of muscles, because they are target organs for growth-promoting treatment with anabolic steroids. In different studies, mRNA expression of steroid receptors was quantified in different androgen dependent muscles, for example, neck muscle, hind limb muscle or shoulder muscle. These studies show that all analyzed muscle types express the androgen receptor and that ERα is the main isoform of the estrogen receptor, pointing out that estrogens mainly act via ERα in muscle tissue.[25,26] Pfaffl *et al*. quantified the mRNA expression of steroid receptors in 10 different parts of the gastrointestinal tract. In all gastrointestinal parts, the expression of each receptor could be quantified, whereas apart from ileum and rectum, ERα is the main expressed isoform of the estrogen receptor.[25,30] There are many other tissues expressing steroid hormone receptors, like liver, kidney, spleen, thymus, bone, skin, lung, and blood leukocytes,[21,22,25,27,31,32] and therefore all these tissues are also potential candidates for the analysis of gene expression biomarkers for treatment with anabolic steroids.

Analytical methods

There are different methods available for the quantification of gene expression. The main difference between the methods is the amount of quantifiable genes. Up to now in most studies where a broad range of gene expression changes shall be quantified, microarray technology is the method of choice. With these arrays it is possible to screen for the expression of all known mRNAs. A holistic approach for gene expression analysis is RNA Sequencing (RNA-Seq), a new method which sequences the whole transcriptome of a biological sample and thereby quantifies all RNA sequences present. This method is very sensitive because only one transcript of a gene is detectable.[33,34] In most of these experiments, gene expression changes are validated by single gene assays using RT-qPCR. Another way for finding candidate genes for biomarker development is the so-called candidate gene approach. Within this approach, candidate genes are determined by screening the actual literature for physiological changes caused by the specific treatment and thereby identifying key factors of these processes, whose expression is then quantified using RT-qPCR. In the presented experiments 30–45 genes were chosen for each tissue using this candidate gene approach. In our lab, the method of RT-qPCR is well established and we are working according to the MIQE guidelines.[35]

Effects of androgens on human hair follicle

Developing gene expression biomarkers for the detection of the abuse of anabolic steroids in human sports is a big challenge, due to dependence on tissues that are available from humans in a non-invasive form. One promising tissue is human hair root cells as it is already known that steroid hormones, especially androgens, act via the dermal papilla cells by influencing different growth factors and enzymes.[36]

In a first *in vitro* pilot study, female and male human hair follicle dermal papilla cells were treated with stanozolol, a xenobiotic androgen. Cells were harvested at different time points after treatment, RNA was extracted, and gene expression analysis was done via RT-qPCR for quantifying 13 different genes. Genes like the androgen receptor (AR), the apoptosis factors Fas receptor (FasR) and caspase 8, the fibroblast growth factor FGF7 and the 5α-Steroidreductase SRD5A2 were detected as significantly regulated, whereas only AR and FasR were significantly regulated in both female and male cells and the other genes were significantly regulated either in female (FGF7, Caspase 8) or in male cells (SRD5A2).[37] In another study, the expression of different genes in plucked hair follicle samples was quantified in female and male hair follicles and also in a small number of hair samples from weightlifters under doping conditions. Thereby no gender dependent differences in gene expression could be quantified. Hair follicles from weightlifters under doping conditions showed significant differences in the expression of the glucocorticoid receptor and the fibroblast growth factor FGF7.[38] These results indicate that it may be possible to find differences in gene expression of hair follicles between treated and untreated individuals and future experiments have to show if more genes are regulated by treatment with anabolic steroids. It has to be proven if these changes could act as specific gene expression biomarkers, in order that abuses can be uncovered.

Effects of androgens on primate muscle and blood cells

As already mentioned, the decrease of the endogenous production of anabolic steroid hormones (mainly estradiol and testosterone) after menopause has a negative effect on bone mineral density leading to osteoporosis.[6] Another problem is sarcopenia, the loss of muscle mass also caused by the decrease in estradiol and testosterone levels. The standard therapy is the replacement of

testosterone or estadiol but these therapies show negative side effects like an increased risk of cancer.[39–41] Alternatively, patients can be treated with selective androgen receptor modulators (SARM) or selective estrogen receptor modulators (SERM). SARM and SERM are synthetic molecules which bind to the steroid hormone receptors exhibiting predominantly tissue selective effects.[42] As these substances positively affect bone density and muscle mass, the risk of misuse in sports and animal husbandry is always present. Therefore a study was designed to compare the effects of a new SARM with those of natural testosterone on gene expression in muscle tissue and whole blood. In this study cynomolgous monkeys were treated either with two different doses of the SARM or with natural testosterone.[43,44] Significant differences in gene expression could be measured for cathepsin L, calpain 3, and IGFBP3 in biopsies of *musculus quadriceps* 90 days after treatment.[44] In whole blood, changes in gene expression could be observed for IL-15 and TNFR2 16 days after treatment started and for IL-12b, IL-15, CD30L, FasR, TNFR1, and TNFR2 90 days after treatment started.[43] In both tissues, no different effects of the SARM and testosterone could be identified.[43,44]

Effects of steroid hormones on bovine tissues

In animal husbandry, steroid hormones are administered as growth promoters as they lead to lean meat due to nitrogen retention and a decrease in total body fat.[4] In the EU, the use of growth promoters is forbidden due to negative side effects of hormone residues for the human consumer.

For the identification of gene expression biomarkers in animals, almost all tissues are available at slaughter. Reproductive organs like uterus, ovary, and vaginal epithelial cells in female animals and testis and prostate in male animals are primary-hormone-dependent tissues and are therefore promising organs to identify physiological effects on the level of transcription.[23,24,26,45] But also other organs like liver, muscle, or blood seem very interesting for the identification of gene expression biomarkers. In two different *in vivo* studies, heifers were treated with steroid hormones and different tissue samples were taken either at slaughter or during treatment time (blood and vaginal smear). In the first study (study 1) the effect of Ralgro® (Zeranol) and Finaplix H® (Trenbolone Acetate) on the expression of different genes in muscle, liver, and uterus was investigated. In a second study (study 2) nine Nguni heifers were treated with Revalor H®, a combination of Trenbolone Acetate plus Estradiol. Blood and vaginal smear samples were taken during treatment time and uterus, ovary, and liver samples were taken at slaughter. In these tissues, gene expression changes were analyzed and compared with nine untreated control animals.

Interesting target genes were chosen by screening the actual literature for steroidal effects on the physiology of the specific organs and their expression was quantified using RT-qPCR. Gene expression changes were statistically analyzed between untreated controls and treated animals.

Effects of steroid hormones on reproductive organs

The effect of anabolic treatment on gene expression in uterine tissue of treated heifers was analyzed in both studies. In the first study, 11 out of 29 tested genes were significantly regulated either by Ralgro® or by Finaplix H® whereas only the apoptosis regulator FasL was regulated by both treatments.[26] In the second study, the analyzed uterus endometrial tissue was separated between uterus horn and uterus corpus. In the uterus horn, 9 genes were found to be differentially regulated; in the uterus corpus 12 regulated genes were identified.[23] Only four genes showed regulations in both regions of the uterus: the androgen receptor, the bone morphogenic factor 4, the apoptosis factor caspase 3, and the complement factor 7.[23] These genes could act as first biomarker candidates for uterine endometrium. In the same study, the effects of Trenbolone Acetate plus Estradiol on gene expression in bovine ovary were analyzed and 22 significantly regulated genes could be identified.[23] This indicates that the ovaries are more promising for biomarker development than the uterine endometrium. A third reproductive organ that has been taken into account in this study was vaginal smear containing vaginal epithelial cells. This was a really new approach because there are no publications available regarding gene expression in bovine vaginal smear. However, gene expression assays could be established for 27 candidate genes and 13 of them could be identified as significantly regulated.[24] Vaginal smear seems to be a promising tissue for biomarker development, because it is available in a non-invasive way from the living animal and so controls could also take place directly on animal farms and not only at the slaughter house.

These results indicate that reproductive organs are promising tissues for the development of gene expression biomarkers for anabolic steroid hormones. But the disadvantage is that the use of these biomarkers is dependent on gender. Due to that, other tissues showing physiological effects caused by treatment with anabolic steroids should been taken into account.

Effects of steroid hormones on other bovine tissues

One interesting tissue for gene expression analysis is muscle, because it is one of the primary physiological target tissues of treatment with growth promoters. In the study regarding the effects of Ralgro® (Zeranol) and Finaplix H® (Trenbolone Acetate) on gene expression in different tissues, the effects on two different muscles (neck muscle and hind limb muscle) were quantified. Treatment with Finaplix H® significantly influenced 6 genes in the neck muscle and also 6 genes in the hind limb muscle, whereas only hexokinase and lactate-dehydrogenase, key enzymes in glycolysis, were regulated in both muscle types. Ralgro® influenced the expression of 3 genes in the neck muscle and 4 genes in the hind limb muscle whereas only IGF-1 was significantly up-regulated in both tissues.[26] These three genes could act as potential biomarkers for the different treatments.

The liver plays an important role in steroid metabolism. It is involved in hormone decomposition and in the production of cholesterol, the precursor for all steroid hormones. Therefore liver is also an interesting target organ for analyzing the effects of steroid hormones on its transcriptome. It could be shown that Finaplix® influenced the expression of 9 out of 18 measured genes and Ralgro® influenced 4 genes on the level of transcription, whereas only the apoptosis regulator FasL and the insulin receptor α (IRα) were influenced by both treatments.[26] This indicates that androgens affect the liver in a higher manner than estrogens. The combination of both Estradiol plus Trenbolone Acetate, influenced the expression of 11 out of 4 analyzed genes in the liver of heifers.[31] IRα that was already regulated in study 1 also showed significant changes in gene expression caused by the treatment, and thus it is a promising biomarker candidate. In study 2, the expression profile of microRNA (miRNA) was also regarded in the liver. miRNAs belong to the class of non-coding small RNAs and

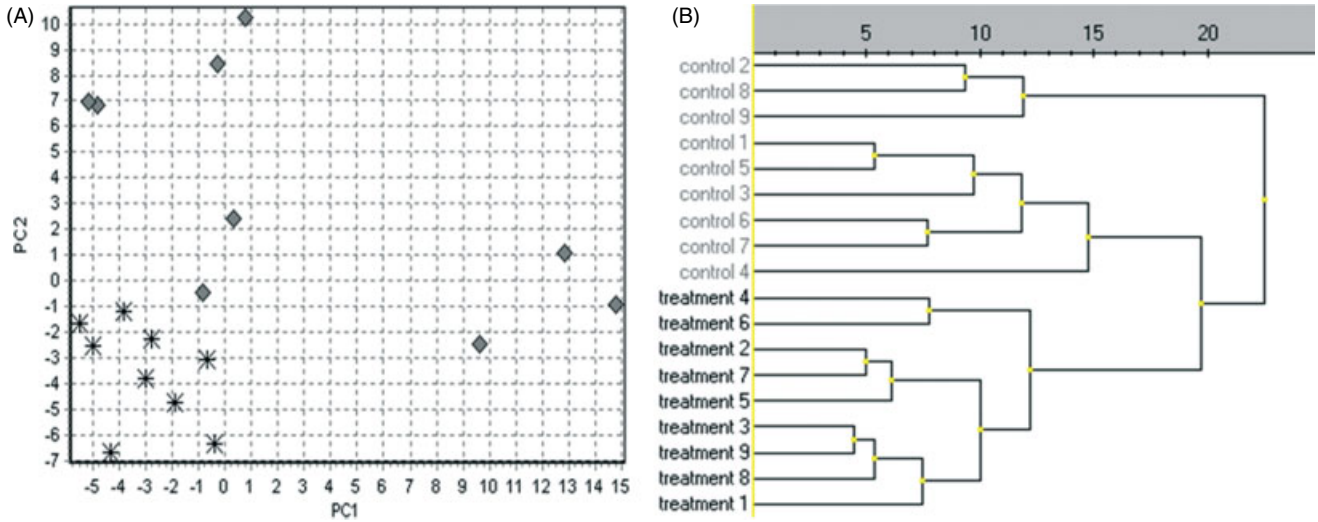

Figure 1. PCA and HCA of results obtained from vaginal smear combined with results obtained from blood. (A) PCA: grey diamonds represent samples of untreated controls, black stars represent samples of treated animals; (B) HCA.

are approximately 22bp of length. They are part of the post-transcriptional regulation of different important physiological processes like differentiation, proliferation, and apoptosis by inhibiting translation or degradation of specific mRNAs.[46] In molecular medicine, for example, cancer research, miRNAs have shown to be specifically expressed in different diseases and tissues and can so act as potential biomarkers.[47–49] There is little knowledge about the expression and the physiological function of miRNA in cattle. Thus finding miRNA biomarker candidates by screening the literature for interesting miRNAs is not a promising approach. Therefore a holistic approach analyzing many miRNAs in one run was taken. miRNA expression of three randomly picked samples from each treatment group (untreated control and treated animals) was analyzed using a ready-to-use PCR Array with 730 human miRNAs. This Array was chosen, because no such possibilities for bovine miRNA exist and it is known that miRNAs are highly conserved between the different species.[46] Promising significantly regulated miRNA candidates were then analyzed in all 18 samples (9 untreated controls and 9 treated animals) using single assay miRNA RT-PCR. From the 730 quantified miRNAs, 22 were significantly up-regulated and 14 were significantly down-regulated whereas 7 additional miRNAs showed a trend to up-regulation and also 7 additional miRNAs showed a trend to down-regulation. From these regulated miRNAs, 11 were quantified with single assay PCR. They were chosen by their fold regulation, their physiological relevance and their homology to bovine miRNA. Five of these genes showed significant regulation regarding all 18 samples and these miRNAs can act as first miRNA biomarker candidates for treatment of heifers with Revalor H®.[46]

As in human sports, blood will also be an interesting matrix for biomarker screening in farm animals, because blood can be taken from the living individual in a non-invasive form and so controls could take place already at the farms and not only at the slaughter house. The effects of Trenbolone Acetate plus Estradiol (Revalor H®) on gene expression in whole blood of heifers were investigated. Eleven out of 36 tested genes showed significant changes, whereas only the glucocorticoid receptor (GRα) and interleukin 1α (IL-1α) were significantly regulated at more than one sampling time point.[32]

The usefulness of biostatistical tools for the development of gene expression biomarkers

All these results indicate that it will be hardly possible to detect one or two specific biomarkers whose expression will be changed under treatment with steroid hormones. But the identification of a pattern of regulated genes seems to be auspicious. The most important question is how to extract the needed information from these data. There are different biostatistical methods for dimensionality reduction available to represent those sets of data in far fewer dimensions.[50] These methods have to be combined with pattern recognition technologies to identify and visualize the desired information.[18]

To take more than three genes into account, multivariate analysis methods like principal components analysis (PCA) or hierarchical cluster analysis (HCA) are required. Principal components analysis reduces multidimensional data sets to lower dimensions called 'principal components'.[51,52] Each analyzed sample will be represented by one spot which results from diminishing all significantly regulated genes of one specific sample to two principal components. This method has effectively been employed to visualize a treatment pattern in primate blood, bovine liver, uterus, ovaries and vaginal smear.[23,24,31,32,43,46] In uterine endometrium and ovary, PCA results obtained from both tissues in combination showed a better separation between the groups than by employing the data obtained from each individual tissue.[23] In bovine liver, the combination of mRNA and miRNA data also resulted in a better visible separation between the treatment groups.[46] In bovine vaginal smear, it could be observed that the incorporation of all analyzed genes showed better separation than only regarding significantly regulated genes.[24] In combination with the results obtained in blood cells, an effective visualization of a treatment pattern could be achieved (Figure 1A). These findings indicate that PCA is a good tool for pattern recognition in gene expression biomarker research.

A further promising method for the visualization of treatment patterns in these studies is hierarchical cluster analysis (HCA). Using HCA, the hierarchical order is represented by a tree dendogram in which related samples are more closely together than samples that are more different.[52] The results obtained in vaginal smear

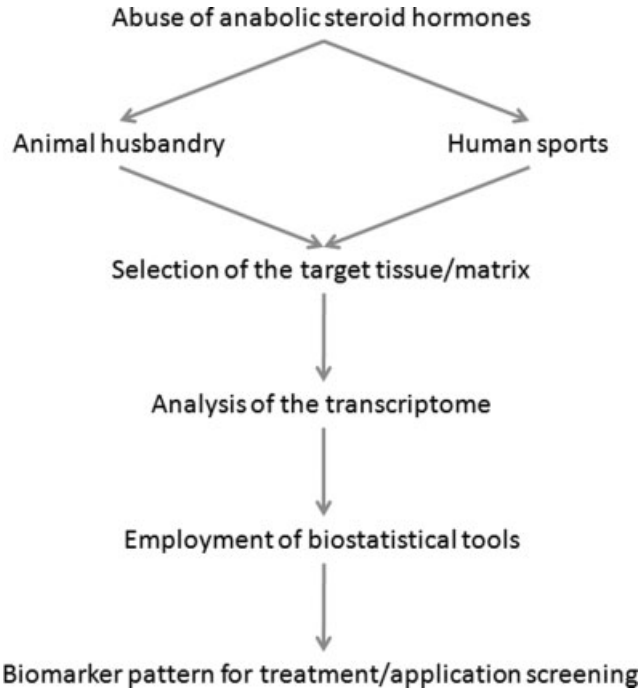

Figure 2. Workflow of the transcriptomic approach to find gene expression biomarkers to trace the abuse of anabolic steroids.

were additionally analyzed using this method resulting in a tree where the treated or the untreated samples respectively are close together and the group of treated samples is separated from the group of untreated samples.[24] Here again the combination with the results of vaginal smear and blood in one HCA resulted in a clear separation of treated and untreated animals (Figure 1B).

Due to high biological variance between individuals caused by environmental conditions or genetic diversity that can be observed by the high spread of the control animals in PCA,[53] a higher number of control animals will be required in future experiments.

Conclusions

All these results indicate that the use of transcriptomics is a promising way to develop new screening methods for the detection of the misuse of anabolic steroids based on physiological changes caused by these substances. Figure 2 displays the workflow for a successful identification of gene expression biomarkers to screen for the abuse of anabolic agents. Most of the presented studies were performed in cattle, as the abuse of growth promoters is also a problem in animal husbandry. The results obtained in human hair papilla cells or in primate and bovine blood indicate that this approach is also a promising way to develop new screening methods useful in doping control. The choice of the target tissue or matrix for humans is a big challenge, because the analyzed tissue/matrix has to been taken in a non-invasive form. Body fluids like blood or urine, which are already used in doping control, will be good candidates. The influence of steroid hormones on blood cells in cattle and primates were already shown in our lab. A future challenge will be to establish an RNA extraction method for urine samples and afterwards analyze gene expression.

Another important observation is that there are no genes regulated in all analyzed tissue. This indicates that each tissue has to be analyzed separately and that gene expression biomarker patterns will be tissue specific.

For the identification of more potential candidate genes, a holistic approach for gene expression analysis will be helpful. A very new approach to screen for changes in gene expression is RNA-Sequencing (RNA-Seq). With this method the whole transcriptome of a biological sample is sequenced and the quantity of each RNA sequence present can be determined. This method is very sensitive because only one transcript of a specific gene is detectable.[33,34]

Even with this method, it seems hardly possible to identify one specific biomarker; rather will there be a pattern of genes whose expression is influenced by treatment with anabolic steroid hormones. Using biostatistical tools, like PCA or HCA the identification of a treatment specific expression pattern seems promising. Another possibility is to combine the transcriptomic approach with other -omic technologies, like proteomics and metabolomics to find more biomarker candidates on other biological levels.[18]

A future challenge will be the detection of gene expression biomarkers for newly designed substances, hormone cocktails, the illegal use of erythropoietin, or other kinds of manipulation like gene or blood doping.

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