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Analysis of the effects of androgens and training on myostatin propeptide and follistatin concentrations in blood and skeletal muscle using highly sensitive Immuno PCR

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Abstract

Myostatin propeptide (MYOPRO) and follistatin (FOLLI) are potent myostatin inhibitors. In this study we analysed effects of training and androgens on MYOPRO and FOLLI concentrations in blood and skeletal muscle using Immuno PCR. Young healthy males performed either a 3-month endurance-training or a strength-training. Blood and biopsy samples were analysed. Training did not significantly affect MYOPRO and FOLLI concentrations in serum and muscle. To investigate whether total skeletal muscle mass may affect circulating MYOPRO and FOLLI levels, blood samples of tetraplegic patients, untrained volunteers and bodybuilders were analysed. MYOPRO was significantly increased exclusively in the bodybuilder group. In orchiectomized rats MYOPRO increased in blood and muscle after treatment with testosterone. In summary our data demonstrate that moderate training does not affect the concentrations of MYOPRO to FOLLI. In contrast androgen treatment results in a significant increase of MYOPRO in skeletal muscle and serum.

Keywords:

Immuno PCR, Myostatin, Myostatin propeptide, Follistatin, Doping, Myostatin Inhibitor, Androgens

Introduction

Great progress has been made over the past years by means of innovative molecular techniques that have led to the discovery of new growth factors involved in the regulation of muscle mass. These findings provide new starting points to understand the mechanisms involved in the adaptation of skeletal muscle to exercise training. One of these newly identified growth factors is myostatin (MSTN), a member of the transforming growth factor-β family of proteins. MSTN was first discovered by McPherron et al. (1997) who showed that a phenotype of exaggerated muscle hypertrophy is correlated with mutations in the MSTN gene. Such naturally occurring knockout mutations of MSTN have been described in animals (Grobet et al. 1997) and in a human child (Schuelke et al. 2004). Blocking of the MSTN signalling transduction pathway by specific inhibitors and genetic manipulations has been shown to result in a dramatic increase of skeletal muscle mass (Bogdanovich et al. 2002; Whittemore et al. 2003; Bogdanovich et al. 2005). This can be achieved either by overexpression of myostatin propeptide (MYOPRO), follistatin (FOLLI) or by the use of specific blocking antibodies. New understanding of the role of MSTN gene expression in growth and development, along with research into the structural and functional characteristics of the MSTN protein, has offered researchers several potential methods to manipulate this signalling pathway. So far, several proteins (e.g., FOLLI, mutant activin type II receptors, and MYOPRO) have been demonstrated to act effectively as MSTN signalling blockers both *in vitro* and *in vivo*. Knocking out of the activin IIB receptor (ACT IIB) for example resulted in a mouse phenotype very similar to MSTN knockout mice (Lee & McPherron 2001).

In principle, blocking of MSTN signalling can be achieved by three different pharmacological strategies. First, there is the possibility to block MSTN gene expression. Knocking out the gene and breeding of transgenic animals can achieve this. Such strategies are particularly interesting for agricultural applications. Another possibility is to inactivate the MSTN gene by

viral-based gene overexpression and antisense technologies (Furalyov et al. 2008; Haidet et al. 2008; Liu et al. 2008). Secondly, the MSTN signalling can be inhibited by blocking the synthesis of the MSTN protein (Huet et al. 2001). A third strategy to inhibit MSTN signalling is to block its receptor. This can be achieved by small molecules or by specific blocking antibodies. Recently, the first results of clinical trials using such inhibitors have been published (Wagner et al. 2008). MSTN inhibitors can potentially be used for agricultural applications, treatment of muscle diseases, treatment of muscle atrophy and metabolic disorders such as obesity and type 2 diabetes (Khurana & Davies 2003; McPherron & Lee 2002; Amthor 2009).

Unfortunately, drugs or genetic manipulations with the ability to modulate MSTN signalling may also have the potential to enhance physical performance in athletes and therefore represent a new class of doping substances. To identify such manipulations knowledge about the regulation of MSTN and proteins able to regulate MSTN activity under different physiological conditions is essential. However the concentrations of many of the members of the MSTN signal transduction pathway are very low, especially in biopsy samples, consequently a highly sensitive detection system is necessary. Therefore, our aim was to use real-time Immuno-PCR (IPCR, Imperacer®) as a technique for antigen detection, which combines the molecular recognition of antibodies with the highly sensitive DNA amplification capability of PCR (Adler, Wacker & Niemeyer 2003; Niemeyer, Adler, Wacker 2005). The procedure is similar to conventional enzyme-linked immunosorbent assays (ELISA) but is more accurate and sensitive and therefore allows the detection of very small amounts of DNA-coupled detection antibody. IPCR and related techniques are highly suitable to detect protein fingerprints with extremely high sensitivity (Niemeyer, Adler & Wacker 2005) and have been successfully adapted and validated as a routine method for quantitative detection of a novel anti-cancer drug for application in clinical studies of pharmacokinetics in human serum (Adler et al. 2005).

Here we describe the development of highly sensitive Imperacer® assays for MYOPRO and FOLLI. Using this technique MYOPRO and FOLLI expression levels were studied in serum and skeletal muscle biopsies under training conditions in young healthy man. The effect of total muscle mass on serum concentrations of MYOPRO and FOLLI was studied in blood samples from tetraplegic patients, untrained volunteers and bodybuilders. To investigate the effect of androgens, protein expression of MYOPRO and FOLLI was studied in serum and skeletal muscle of DHT treated castrated male rats.

Materials and Methods

ANTIBODIES AND RECOMBIANT PROTEINS

ELISA/Western Blot

Antibodies: Follistatin antibodies AF669 and BAF669 (R&D Systems), Myostatin Propeptide, antibodies RD 183057050 and RD-1057 (R&D Systems). Myostatin antibodies Myo 1 and Myo2 (Immunodiagnostik AG Bensheim). Detection limit of the Myostatin ELISA is 0,273 ng/ml, LOD: limit of determination: 0,6 ng/ml, LOQ: limit of quantitation: 1,15 ng/ml , Inter assay CV: < 15% Intra assay CV: < 10%

Recombinant proteins: Myostatin GDF-8 (mouse) 788-G8 (R&D Systems), Follistatin : (human) 4708-20, (BioVision), Myostatin Propeptide: Human MYOPRO, recombinant protein; Cat-no. RD172058100 (BioVendor).

Immuno PCR:

Capture antibody for Myostatin Propeptide: Anti-Human MYOPRO, Mouse Monoclonal Antibody; Cat.-no. RD-1057 (BioVendor) Clone 6E8.

Recombinant Myostatin Propeptide: Human MYOPRO, recombinant protein; Cat-no. RD172058100 (BioVendor).

Capture antibody for Follistatin: Human FOLLI Cat.-no. AF669, (R&D Systems)

Recombinant Follistatin: Human FOLLI, recombinant Protein; 4708-20 (BioVision). Standardized serum (BISEKO) for spiking, Source: Biotest.

IMMUNO PCR

Immuno PCR (Fig 1 A) was performed using the Chimera Imperacer® kits Human FOLLI Immuno PCR Assay Catalog Number 11-000 kit-R and Human Myo-Pro Immuno-PCR Assay Catalog Number 11-039 kit-R. The following listed additional antibodies and recombinant proteins were used:

A flow chart of an Immuno PCR assay is shown in Fig. 1B. A monoclonal anti-FOLLI antibody (“AF669”, R&D Systems) or monoclonal anti MSTN propeptide antibody (RD-1290 (human) R&D Systems) was immobilized onto a microplate surface and the plate was blocked against unspecific binding by incubating with Chimera Imperacer® kit blocking buffer over night at 4°C . Previous to the blocking step, standards and samples were mixed with a solution of an antibody-DNA detection conjugate specific for FOLLI (CHI-Foli) or MSTN propeptide (CHI-Myo) for combined incubation in siliconized cups. Subsequently, the mixture was incubated in each blocked well, followed by a washing step to remove any unbound sample material and antibody-DNA conjugate. Finally, a PCR-mastermix was added to the wells.

Real-time PCR was carried out using a dual-labelled probe. The probe contains a fluorescence dye (FAM) and a quencher. While the DNA-polymerase activity of the enzyme elongates the PCR-primer during the synthesis of the novel complementary DNA strand, the probe will be sterically altered, thereby inducing fluorescence for each amplified DNA strand. Signal read out of a real-time Immuno PCR experiment was measured in a Stratagene p3500 cycler. Amplification was performed using the following listed PCR program: PCR-program for real-time 5 min 95°C 1x, 12 sec 95°C, 30 sec 50°C, 30 sec 72°C, 45x.

CALCULATIONS OF RESULTS

The real-time PCR-cycler records the increase of the normalized fluorescence signal (dR) for each cycle during DNA amplification. Subsequent to the run, the software of the instrument applies an automatic baseline correction. In the next step, the software automatically calculates the threshold cycle (Ct), which represents the first PCR cycle at which the reporter signal exceeds the signal of a given uniform “threshold”, manually set in the phase where the signal increases linearly (typically 100-000). A half-logarithmic plot of log dR against cycle number is used to choose the correct threshold value. To render an easy comparison of data obtained from real-time Imperacer® and conventional ELISA, the problem has to be circumvented that Ct values are inversely proportional to antigen concentrations (the negative control NC has the highest numerical value) while ELISA signals are directly proportional to antigen concentrations (NC has the smallest numerical value). Therefore, we calculated delta Ct values by subtracting the Ct values obtained for each signal from the total number of cycles carried out in the experiment (45-Ct). For each sample and/or standard analysed in duplicate the mean values and standard deviation of delta Ct was calculated. For quantification, the delta Ct of the calibration curve standards was plotted against the log concentration and a linear regression for concentrations or a non-linear regression (e.g. quadratic fit) was carried out. The resulting equation was used for the determination of the antigen concentration in unknown samples.

SUBJECTS:

Training experiment

The study was approved by the ethic committee of the German Sport University. Experiments were conducted according to the Declaration of Helsinki. All subjects gave their written consent to participate after being informed about the specific risks and the studies protocol as well as a medical examination. 33 male sport students (no structured, specific strength and endurance training for at least 6 months before beginning the study) with an average (±SD) age of 22.2 (1.8) yr, height of 183.2 (5.9) cm, and body weight of 77.8 (8.0) kg

participated in this study. Subjects were randomly assigned to either an endurance-training group E (N = 11), a strength-training group S (N=11) or a control group C (N=11). 27 of these students, belonging to E (N = 8), S (N=10) and C (N=9) finished the study and were analysed.

Muscle mass experiment

The anthropometric data of the analysed bodybuilders and tetraplegic subjects are summarised in Tab. 2. The control group was composed of non specifically trained male sport students as described above under training experiment.

TRAINING PROTOCOL:

According to medical history and examination all subjects were healthy. They participated in various physical activities predominantly in sport games such as soccer, handball and basketball. A systematic and regular strength and endurance training within the last 6 month was prohibited. All participants were demanded to continue their regular lifestyle including physical activity and nutrition habits during the study.

The participants of the groups trained three days a week, with a two-day rest period between the training units. The training intensity for S was specified on the basis of the one repetition maximum (1RM) test, which had been determined in the first training unit. The intensity corresponded to a training weight of 70-80% of the one repetition maximum (1RM) and a repetition number from eight to ten per session, with 2 minutes rest between the sets. The subjects performed a complete body workout including leg extension, leg curl, chest press, leg press, leg pulley, seated row, lateral raise, crunches and hyperextension. Each motion cycle (concentric - eccentric) persisted of three to four seconds. In order to maintain an appropriate training stimulus for improving strength abilities during the training period, the subjects were instructed to adjust their training weight, if ten repetitions of the original weight could be completed without problems. E took place in form of a 45-minute run for each

training unit. The training intensity was controlled by heart rate (HR) measurement. Study participants trained at 80% of their HR that corresponded to the aerobic/anaerobic threshold. After 8 weeks of training an intermediate testing of the strength and endurance group took place to potentially modify training intensity. S carried out a one repetition maximum test and E fulfilled a treadmill test that included lactate and HR measurement. The final tests were developed identically to the entrance examinations and took place at the beginning of week twelve. It was ensured that the final tests were conducted in the same sequence and at the same time as the entrance examinations.

PHYSICAL FITNESS TESTING:

Physical fitness tests were carried out before and after the training period, after a standardized resting period of 2 days. Isometric strength abilities of knee extensior muscles were measured in a well-standardized position sitting upright with a knee flexion of 120° in a leg extension machine (Gym 80, Gelsenkirchen, Germany). The measurement took 10 seconds of maximal isometric strength output. The subjects were asked to do a 1RM described in Bell et al. (2008) to find the correct intensity of 70-80 %.

Endurance testing:

Endurance was tested on a treadmill (Woodway GmbH, Weil am Rhein, Germany). The entrance velocity was set at 7.0 km/h (1.94 m/s). There upon the load was increased every four minutes by 1.5 km/h (0.42 m/s) till exhaustion. Oxygen uptake was analysed by an open breath-by-breath system (ZANG80, Oberthulba, Germany). Heart rate was recorded with polar Vantage XL (Polar Electro, Kempele, Finnland). Capillary blood lactate was collected in sample tubes from the ear lobe and analysed with Biosen c-line (EKF-diagnostic, Barleben, Germany) before the test and at the end of every stage.

Tissue collection:

Muscle biopsies were obtained 3 to 5 days before starting the exercise period and again after the exercise period. There were at least two days rest between the last exercise bout and the biopsy. Food intake was standardized one day before the biopsies. All individuals underwent the first and the second biopsy at identical daytime. Muscle samples were taken by a Bergstroem needle from the middle portion of the right vastus lateralis muscle at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. After removal, muscle tissue was immediately frozen in isopentane with -80 °C, then in liquid nitrogen and finally stored at -80°C for analysis.

After an overnight fast venous blood samples were drawn from an antecubital vein early in the morning. Blood samples were centrifuged at 3000 rpm for 10 minutes and serum was stored at -80°C for later analysis.

Protein preparation from biopsy samples:

Pooled frozen biopsy samples were powdered and homogenized in buffer (623,5 mM Tris/EDTA, pH 8) containing enzyme inhibitors (5mg/ml aprotonin, 5mg/ml leupeptin, 1mg/ml pepstatin-A, 5mg/ml antipain, 100 mM pefac in 0.5M EDTA pH 8). Protein concentrations were determined by the method of Lowry (Dc Protein Assay, Bio-Rad). Dilutions of proteins were made in sample dilution buffer.

Animal Experiment

Male Wistar rats (130g, age 7 weeks) were obtained from Janvier Laboratories (Le Genest St. Isle, France) and were maintained under controlled conditions of temperature (20°C $\pm$ 1, relative humidity 50-80%) and illumination (12 h light, 12 h dark). All rats had free access to a standard rat diet (SSniff R10-Diet, SSniff GmbH, Soest, Germany) and water. They were maintained according to the European Union guidelines for the care and use of laboratory animals. The study was undertaken with the approval of the regional administration of the governmental body.

The Hershberger assay was performed according to the guidelines of the rodent Hershberger assay (Yamasaki et al. 2003). The rats were orchiectomised under anesthesia (Rompun / Ketanest). After 7 days of endogenous hormone decline, the animals were randomly allocated to treatment and vehicle groups (n = 6). For subcutaneous (s.c.) administration DHT was dissolved in ethanol and diluted in corn oil. The animals were treated once a day s.c. with DHT (1 mg/kg BW/day) for 12 days.

Rats were sacrificed after completion of the 12-day treatment period. Following removal, wet weights of the prostate, seminal vesicle and levator ani muscle were determined.

The data of tissue weights are presented as mean ± standard error of the mean (SEM).

STATISTICAL ANALYSIS

Statistical analyses were performed using the SPSS Statistical Analysis System, Version 12.0.

All data are expressed as arithmetic means with their standard deviations. Statistical significance of differences was calculated using one-way analysis of variance (ANOVA) followed by post hoc Tukey HSD test where appropriate. Statistical tests were used for comparisons between groups and statistical significance was established at $P<0.05$.

Results

Development of high sensitive Imperacer® for MYOPRO and FOLLI

To develop a highly sensitive assay for the detection of MYOPRO and FOLLI in serum and tissue samples several antibodies were tested. Using the antibodies mentioned in materials and methods we were able to develop functional ELISA's. Based on these ELISA's functional Imperacer® assays for FOLLI and MYOPRO were developed using the principle of Immuno PCR (Fig. 1 A). The selectivity of the used antibodies was tested by western blotting in human serum protein (Fig. 2 A). The sensitivity of the Imperacer® based assays was superior compared to the corresponding ELISA's (Fig. 2 B and C). Limit of detection, limit of quantitation and the intra- and inter-assay coefficients of variation are provided in TAB. 1.

Effects of training on the expression of MYOPRO and FOLLI in the serum samples of trained athletes

To investigate the effects of training on the serum concentration of MYOPRO and FOLLI, three groups of volunteers were trained for three months as described in material and methods. Effects of training on strength and endurance are shown in Fig. 3 A and B. It is clearly visible that strength increased significantly in ST (Fig 3 A), whereas endurance increased in ET (Fig. 3 B). FOLLI and MYOPRO concentrations in the serum of the volunteers were determined before and after the training period. As shown in Fig. 4 Imperacer® technology allows highly sensitive and reliable measurements of MYOPRO and FOLLI in human serum. Interestingly, all analysed individuals had very stable serum

concentrations of FOLLI (Fig. 4 A). Obviously, neither endurance nor strength training affected the average serum levels of MYOPRO (Fig. 4 C) and FOLLI (Fig. 4 B).

Effects of Training on the expression of MYOPRO and FOLLI in muscle biopsies

To investigate whether the detected serum levels of MYOPRO and FOLLI correlate to protein concentrations in the trained skeletal muscles, biopsies were taken before and at the end of the training period. Using Imperacer® the concentrations of MYOPRO and FOLLI were determined in biopsy samples from the end of the training period. Whereas the concentration of MYOPRO in the biopsies was below the detection limit, FOLLI concentrations could be determined. In agreement with the serum concentrations no training effects were observed (4 D).

Effects of the total muscle mass on the ratio of MYOPRO and FOLLI

Since physical activity in the performed endurance and strength training had no effects on FOLLI and MYOPRO serum and tissue concentration, we were interested whether there may be an alteration by total body muscle mass. Therefore, we collected serum samples from tetraplegic patients and bodybuilders and compared them to trained young healthy male sport students. The detailed personal characteristics of the investigated groups are shown in Tab. 2. The serum levels of MYOPRO were significantly increased in the bodybuilder group (Fig. 5A). In contrast the expression levels of FOLLI remained unaffected (Fig. 5 A). Remarkably, there was no difference between the control group and the tetraplegic people. To verify the data, the serum concentration of total MSTN was determined by ELISA in tetraplegic persons and bodybuilders. Similar to the FOLLI results there was no difference between the two groups (Fig. 5 A). The calculated ratio FOLLI/MYOPRO (Fig. 5 B) was significantly lower in the bodybuilder group.

Effects of testosterone on MYOPRO and FOLLI in serum and m. gastrocnemius of orchiectomised rats

To investigate the effects of androgens on MYOPRO and FOLLI, orchiectomised rats were treated with Dihydrotestosterone (DHT) as described in material and methods. As shown in Fig. 6 A and B prostate weight and Lev ani weight were stimulated by DHT treatment. MYOPRO and FOLLI concentrations in the plasma of the animals were determined by IPCR. In Fig. 6 C it is clearly visible that the FOLLI/MYOPRO ratio is significant lower in the animals treated with testosterone. MYOPRO expression in the m. gastrocnemius is increased in the DHT treated animals (Fig. 6 D).

Discussion

To identify manipulations of MSTN signalling, a promising strategy is to monitor the expression fingerprints of members of the MSTN signalling pathway in different biological samples. Therefore, our aim was to establish a real-time Immuno PCR method ("IPCR", Imperacer®) for the sensitive and reliable detection of MYOPRO and FOLLI. In this study we were able to establish functional ELISAs and Imperacer® assays for FOLLI and MYOPRO. An important result of our investigations is the fact that the overall sensitivity of the developed Imperacer® assays was significantly higher compared to the sensitivity in the corresponding ELISAs (Fig. 2). Immuno PCR is a detection system with superior sensitivity that allows protein detection using small volumes of serum samples (below 30µl). This offers the opportunity to use capillary blood or other biological matrices like saliva for testing. Moreover, using Immuno PCR we determined protein concentrations in skeletal muscle tissue. Analysing muscle biopsies we were able, at least with Imperacer®Folli, to determine

FOLLI concentrations in these tissues (Fig. 4 D). In contrast to conventional techniques, such as ELISA or Western Blotting, Immuno PCR in this case allows quantitative detection.

To investigate individual variability and effects of physical activity on serum concentrations of MSTN, MYOPRO and FOLLI, the impact of a three-month strength and endurance training was analysed. As shown in Fig. 3 training resulted in a significant increase of strength in the resistance trained individuals, whereas endurance training resulted in a right shift of the lactate curve, demonstrating an increase in aerobic capacity. Both training protocols obviously resulted in measurable physiological changes. Analysing the individual MYOPRO and FOLLI levels in the training groups before and after the training period (Fig. 4) revealed that there is only a moderate inter-individual variability with respect to the measured MYOPRO and FOLLI serum levels. In our study the serum concentrations varied between 6 and 10 ng/ml for FOLLI and between 35 and 50 pg/ml for MYOPRO. With respect to FOLLI serum concentrations, data presented in the literature is very controversial. There are several reports analysing human FOLLI serum levels in females. In these investigations the observed levels vary between 57 ng/ml in pregnant women (Rae et al. 2007) and 0.49 ng/mL (Reis et al. 2007) in healthy women. MYOPRO is known to bind and inhibit MSTN in vitro (Thies et al. 2001). This interaction has also been shown to be relevant in vivo (Hill et al. 2002) where about 70% of MSTN in serum is bound to its propeptide. Reports about the typical FOLLI serum levels in healthy young men as well as reports about typical human MYOPRO serum levels are, to our knowledge, not available.

One of the major aims of this study was to identify biomarkers to detect manipulations in the MSTN signalling. Therefore, our finding that the individual ratios of the analysed proteins turned out to be very constant (Fig. 4) and were not affected by training (Fig. 4 B, C) is very important. A biomarker, which is already affected by moderate training, may not be a suitable marker to detect the abuse of MSTN inhibitors. In Fig. 4 A it is visible, that the individual FOLLI serum concentrations are highly constant. In individual samples, taken in a 3-month interval, nearly identical concentrations were determined. The inter-individual variability

seems to be low and much smaller than the intra-individual variability. This observation can be taken as an indication for the reliability of our test system and it demonstrates that the used training protocol does not affect FOLLI serum concentrations. This is in good agreement to the FOLLI concentrations determined in the biopsy samples. FOLLI expression was detectable in the skeletal muscle biopsy samples, but in good correlation to the determined concentrations measured in serum, it was not affected by training. Effects of training on the mRNA and protein expression of FOLLI and MSTN in human muscle biopsies have been reported in several studies. In other studies FOLLI expression in the M. vastus lateralis (Jensky et al. 2007) was not affected by training. Also there are recent studies investigating long term training effects on MSTN and MYOPRO proteins (Hulmi et al. 2009; Kim et al. 2007). In both studies no major effects of long term training on myostatin or its propeptide (Kim et al. 2007) could be detected.

To get an impression whether total muscle mass may affect the expression ratios of the chosen proteins, serum concentrations were determined in individuals, with extreme different muscle. Non-trained, healthy young males, tetraplegic patients and bodybuilders were compared. The main result of these examinations is that there was no significant difference in the average serum concentrations of all proteins between healthy young men and tetraplegic patients (Fig. 5 A). Interestingly, the average serum concentration of MYOPRO was significantly elevated in the bodybuilder group compared to the control and tetraplegic group (Fig. 5 A). This observation could be taken as an indication that the extremely high skeletal muscle mass of bodybuilders affects the circulating MYOPRO levels. However, bearing in mind that there was no difference between the control group and the tetraplegic group (the tetraplegic group had a significantly lower muscle mass than the control group of healthy young men), an additional explanation could be that other factors like the extreme training protocol or the intake of anabolic substances (most likely anabolic steroids) caused the elevated levels. In a recent publication it has been reported, that MSTN levels were significantly higher in older men treated with graded doses of testosterone (Lakshman et al. 2009). A modulation of MSTN activity by anabolic steroids would fit our recent observation

that MSTN expression in cell culture and in animal studies is modulated by androgens and anabolic steroids (Diel et al. 2008a; Diel et al. 2008b). The hypothesis is also supported by our animal experimental data shown in Fig. 6. It is highly visible that treatment with DHT (1 mg/kg/BW/day) increased MYOPRO expression in the m. gastrocnemius (Fig. 6d) and, like observed in the bodybuilder group, the FOLLI/MYOPRO (Fig. 6c) ratio decreased in animals treated with DHT.

Summarising all data shown, we suppose that the FOLLI and MYOPRO Imperacer® assays are promising tools to detect manipulations of MSTN signalling. The sensitivity of the assay is high enough to detect the relevant proteins in small samples (capillary blood or sputum) and in the future, multiplexing and/or polyplexing would allow the simultaneously detection of at least two proteins in a single sample. The serum ratios of the analysed proteins turned out to be very constant and were not affected by physical activity. In contrast, manipulations resulting in an extreme and perhaps unphysiological increase of muscle mass seemed to affect the ratios. Because MSTN signalling seems to be a basic molecular mechanism in skeletal muscle adaptation, the analysis of ratios of members of MSTN signalling may be a strategy to identify anabolic manipulations independent of the substance or technique used.

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References

Adler M, Wacker R, Niemeyer CM, 2003. A real-time immuno-PCR assay for routine ultrasensitive quantification of proteins. Biochem Biophys Res Commun 308: 240-250.

Adler M, Langer M, Witthohn K, Wilhelm-Ogunbiyi K, Schöffski P, Fumoleau P, Niemeyer CM, 2005. Adaptation and performance of an immuno-PCR assay for the quantification of aviscumine in patient plasma samples. J Pharm Biomed Anal 39: 972-982.

Amthor H, Otto A, Vulin A, Rochat A, Dumonceaux J, Garcia L, Mouisel E, Hourdé C, Macharia R, Friedrichs M, Relaix F, Zammit PS, Matsakas A, Patel K, Partridge T, 2009 Muscle hypertrophy driven by myostatin blockade does not require stem/precursor-cell activity.Proc Natl Acad Sci U S A. 106:7479-84.

Bell DR, Padua DA, Clark MA, 2008. Muscle strength and flexibility characteristics of people displaying excessive medial knee displacement. Arch Phys Med Rehabil 89: 1323-1328.

Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS, Khurana TS, 2002. Functional improvement of dystrophic muscle by myostatin blockade. Nature 420: 418-42.

Bogdanovich S, Perkins KJ, Krag TO, 2005. Whittemore LA, Khurana TS. Myostatin propeptide-mediated amelioration of dystrophic pathophysiology. FASEB 19: 543-549.

Diel P, Baadners D, Schlüpmann K, Velders M, Schwarz JP, 2008. C2C12 myoblastoma cell differentiation and proliferation is stimulated by androgens and associated with a modulation of myostatin and Pax7 expression. Mol Cell Endocrinol 291: 1004-1010.

Diel P, Friedel A, Geyer H, Kamber M, Laudenbach-Leschowsky U, Schänzer W, Schleipen B, Thevis M, Vollmer G, Zierau O, 2008. The prohormone 19-norandrostenedione displays selective androgen receptor modulator (SARM) like properties after subcutaneous administration. Toxicol Lett 177:198-204.

Furalyov VA, Kravchenko IV, Khotchenkov VP, Popov VO, 2008. siRNAs targeting mouse myostatin. Biochemistry 73: 342-345.

Grobet L, Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Ménissier F, Massabanda J, Fries R, Hanset R, Georges M. A deletion in the 23 Jensky NE, Sims JK, Rice JC, Dreyer HC, Schroeder ET, 1997. The influence of eccentric exercise on mRNA expression of skeletal muscle regulators. Eur J Appl Physiol 101: 473-480.

Haidet AM, Rizo L, Handy C, Umapathi P, Eagle A, Shilling C, Boue D, Martin PT, Sahenk Z, Mendell JR, Kaspar BK, 2008. Long-term enhancement of skeletal muscle mass and strength by single gene administration of myostatin inhibitors. Proc Natl Acad Sci U S A. 105: 4318-4322.

Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y, 2002. The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J Biol Chem 277: 40735-40741.

Huet C, Li ZF, Liu HZ, Black RA, Galliano MF, Engvall E, 2001. Skeletal muscle cell hypertrophy induced by inhibitors of metalloproteases; myostatin as a potential mediator. Am J Physiol Cell Physiol 281: C1624-1634.

Hulmi JJ,Tannerstedt J, Selänne H, Kainulainen H, Kovanen V, Mero A, 2009. Resistance exercise with whey protein ingestion affects mTOR signalling pathway and myostatin in men. J Appl Physiol 106: 1720-1729.

Jensky NE, Sims JK, Rice JC, Dreyer HC, Schroeder ET, 2007. The influence of eccentric exercise on mRNA expression of skeletal muscle regulators. Eur J Appl Physiol 101: 473-480.

Khurana TS, Davies KE, 2003. Pharmacological strategies for muscular dystrophy. Nat Rev Drug Discov 2: 379-390.

Kim J, Petrella J, Cross J, Bamman M, 2007. Load mediated downregulation of myostatin mRNA is not sufficient to promot myofiber hypertrophy in humans, a cluster analysis. J Appl Physiol 106: 1488-1495.

Lakshman KM, Bhasin S, Corcoran C, Collins-Racie L, Tchitiakova LA, Forlow S, Ledger K, Burczynski M, Dorner a, Lavallie E, 2009. Measurement of myostatin concentrations in human serum: Circulating concentrations in young and older men and effects of testosterone administration. Molecular and Cellular Endocrinology 302:26-32.

Lee SJ, McPherron AC, 2001. Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci U S A 98: 9306-9311.

Liu, CM, Yang Z, Liu CW, Tien P, Dale R, Sun L, 2008. Myostatin antisense RNA-mediated muscle growth in normal and cancer cachexia mice. Gene Therapy 15: 155-160.

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

McPherron AC, Lee SJ, 2002. Suppression of body fat accumulation in myostatin-deficient mice. J Clin Invest. 109: 595-601.

Niemeyer CM, Adler M, Wacker R, 2005. Immuno-PCR: high sensitivity detection of proteins by nucleic acid amplification. Trends Biotechnol 23: 208-216.

Rae K, Hollebone K, Chetty V, Clausen D, McFarlane J, 2007. Follistatin serum concentrations during full-term labour in women-significant differences between spontaneous and induced labour. Reproduction 134: 705-711.

Reis FM, Nascimento LL, Tsigkou A, Ferreira MC, Luisi S, Petraglia F, 2007. Activin A and follistatin in menstrual blood: low concentrations in women with dysfunctional uterine bleeding. Reprod Sci 14: 383-389.

Schuelke M, Wagner KR, Stolz LE, Hübner C, Riebel T, Kömen W, Braun T, Tobin JF, Lee SJ, 2004. Myostatin mutation associated with gross muscle hypertrophy in a child. N Engl J Med. 350: 2682-2688.

Thies RS, Chen T, Davies MV, Tomkinson KN, Pearson AA, Shakey QA, Wolfman NM, 2001. GDF-8 propeptide binds to GDF-8 and antagonizes biological activity by inhibiting GDF-8 receptor binding. Growth Factors 18: 251-259.

Wagner KR, Fleckenstein JL, Amato AA, Barohn RJ, Bushby K, Escolar DM, Flanigan KM, Pestronk A, Tawil R, Wolfe GI, Eagle M, Florence JM, King WM, Pandya S, Straub V, Juneau P, Meyers K, Csimma C, Araujo T, Allen R, Parsons SA, Wozney JM, Lavallie ER, Mendell JR, 2008. A phase I/II trial of MYO-029 in adult subjects with muscular dystrophy. Ann Neurol 63: 561- 566.

Whittemore LA, Song K, Li X, Aghajanian J, Davies M, Girgenrath S, Hill JJ, Jalenak M, Kelley P, Knight A, Maylor R, O'Hara D, Pearson A, Quazi A, Ryerson S, Tan XY, Tomkinson KN, Veldman GM, Widom A, Wright JF, Wudyka S, Zhao L, Wolfman NM, 2003. Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. Biochem Biophys Res Commun 300: 965-967.

Yamasaki K, Sawaki M, Ohta R, Okuda H, Katayama S, Yamada T, Ohta T, Kosaka T, Owens W, 2003. OECD validation of the Hershberger assay in Japan: phase 2 dose response of methyltestosterone, vinclozolin, and p,p'-DDE. Environ Health Perspect 111: 1912-1919.

Legends for Figures

Fig. 1

A. Principle of Immuno PCR.
B. Flow chart showing the individual steps involved in performing an Immuno PCR assay

Fig. 2

Western blot of human serum protein (30µg protein/lane, 4 different samples) demonstrating the binding specificity of the used antibodies.

A. A single band is detected by the respective antibodies (MYOPRO 28kDa, FOLLI 38 kDa)

B. Comparison of the developed MYOPRO (Myo-P) assays to the corresponding ELISA.

C. Comparison of the developed Follistatin assays to the corresponding ELISA.

Fig. 3 Training results

Three groups of volunteers were trained for three months as described in material and methods. Effects of training on strength (Fig. 3 A) and endurance performance (Fig. 3 B) are shown.

A. Measured pre – post differences of isometric strength for knee extensor muscles in newton before and after the training period. * Significant higher increase of isometric strength ($p < 0.05$) in the post test of the strength group.

B. Measured lactate concentrations in blood in relation to running velocity. Measurements were performed before and after the three-month training period. * Significant lower ($p < 0.05$) lactate concentrations all over different running velocities in the post test of the endurance group.

S – Individuals of the resistance training group, E - Individuals of the endurance training group, C - untrained control group.

Fig. 4

Measured serum and tissue concentrations (Vastus lateralis) of MYOPRO and FOLLI in the trained individuals.

A. delta CT values of FOLLI. Shown are the reference (synthetic serum, BISEKO spiked with the respective recombinant proteins) and the determined delta CT values of individuals (each number is one individual) before and after the 3-month training period. The figure demonstrates individual differences in the FOLLI concentrations between the probands, but also that training does not affect the individual FOLLI concentrations.

B. Average FOLLI serum concentrations in the different training groups before and after the 3-month training period. R resistance group baseline, RT resistance group after 3 month training, E endurance group baseline, ET endurance group after 3 month training, C control group, CT control group after 3 months.

C. Average MYOPRO serum concentrations in the different training groups before and after the 3-month training period. R resistance group baseline, RT resistance group after 3 month training, E endurance group baseline, ET endurance group after 3 month training , C control group, CT control group after 3 months.

D. Measured tissue concentrations of FOLLI in biopsies taken from the vastus lateralis muscle of individuals. Average FOLLI tissue concentrations in the different training groups, after the 3-month training period are shown.

Fig. 5

Measured serum concentrations of MYOPRO and FOLLI in untrained healthy young men (Control), tetraplegic patients (TETRA) and body builders (BODY).

6A. Measured serum concentrations of MYOPRO, FOLLI and total myostatin (MYO). MYOPRO and FOLLI serum concentrations were determined by Immuno PCR. Total MYO was determined by a specific ELISA. ND means not analysed.

6B. Calculated ratios of FOLLI/MYOPRO from delta CT values.

* Significant different in comparison to control ($p < 0.05$), T-test

Fig. 6

Effects of Dihydrotestosterone (1 mg/kg /BW) on MYOPRO expression in orchiectomised male rats.

Fig. 6A. Prostate weight

Fig. 6B. Lev. ani weight

Fig. 6C. Calculated ratios of FOLLI/MYOPRO from delta CT values in rat serum

Fig. 6D. A representative western blot of MYOPRO expression in the m. gastrocnemius is shown. The figure demonstrates the mean results of a densitometric analysis of 3 different western blots.

Orchi =orchiectomy, DHT= treatment with Dihydrotestosterone.

* Significant different in comparison to Orchi ($p < 0.05$), T-test

	Average ***intra-assay CV%*** of **measured delta Ct**	Average ***intra-assay CV%*** of **calculated concentrations**	Average ***inter-assay CV%*** of **measured delta Ct**	Average ***inter-assay CV%*** of **calculated concentrations**	Average ***recovery (R_R)*** of **calculated concentrations**	**LOD** *(Limit of detection)*	**LLOQ** *(Lower limit of quantification)*
Myo-Pro	*0.9 ± 1%*	*10 ± 5%*	*1 ± 1%*	*13 ± 12%*	*98 ± 12%*	*2.4 pg/ml*	*6.4 pg/ml*
Folli	*1.9 ± 1%*	*10 ± 6%*	*2.4 ± 2*	*24 ± 13%*	*113 ± 37%*	*40 pg/ml*	*64 pg/ml*

Tab. 1: *Statistical analysis of Imperacer® assay performance*

In a set of two intra- and inter-assay double determinations of a spiked calibration curve for each antigen, the following assay parameters of Imperacer® were obtained:
Assay precision was determined as standard deviation of measured signals (delta Ct) and calculated concentrations (4 parameter fit, logistic model, R2 = 0.99 for all three antigens, respectively) in double determination and represented as percent of average signal (CV%).
Detection limit ("LOD") was calculated according to DIN 32645 by significant difference of 3 x standard deviation of the negative control. Assay recovery of calculated concentrations was determined in % of spiked concentrations. The lowest actual spiked concentration which allowed an recovery >80% was determined as lower limit of quantification (LLOQ).

Tab 2. Anthropometric data of analysed bodybuilder and tetraplegic subjects.

Tetraplegic subjects

Month: Time since the accident leading to tetraplegy
Tetraplegy: First functional nerve segment

Subject	Gender	Age [yr]	Tetraplegy	Month
1	M	18	C3	18
2	M	34	C5	12
4	F	44	C4	12
5	M	53	C7	12
6	F	33	C4	11
7	M	64	C4	15
8	M	33	C5	16

Body Builders

Subject	Gender	Body weight [kg]	Age [yr]	Bench press [kg]
1	M	95	34	180
2	M	92	34	150
3	M	91	28	170
4	M	85	38	145
5	M	86	29	140
6	M	81	33	140
7	M	93	35	150
8	M	90	33	140

Figure

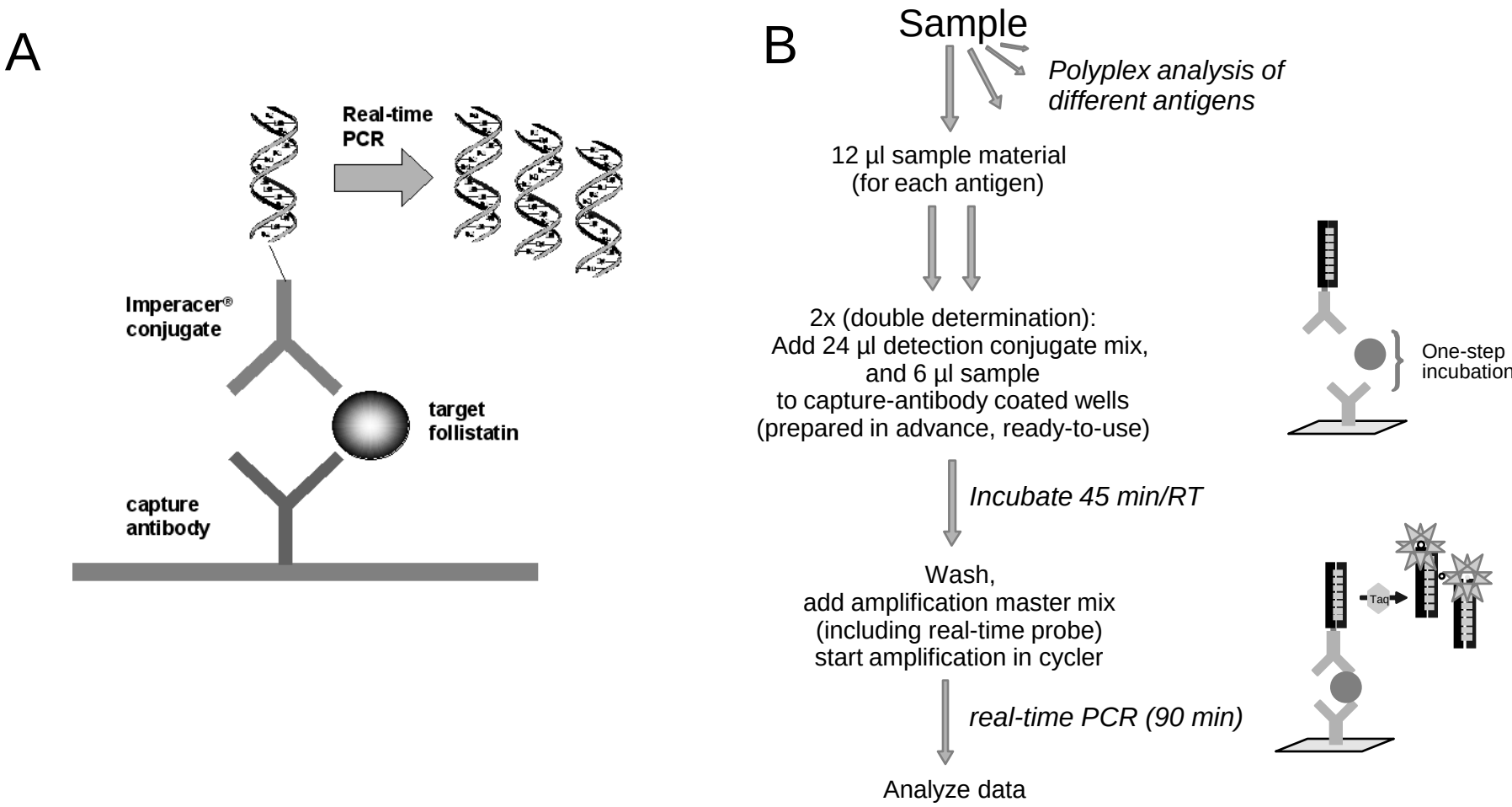
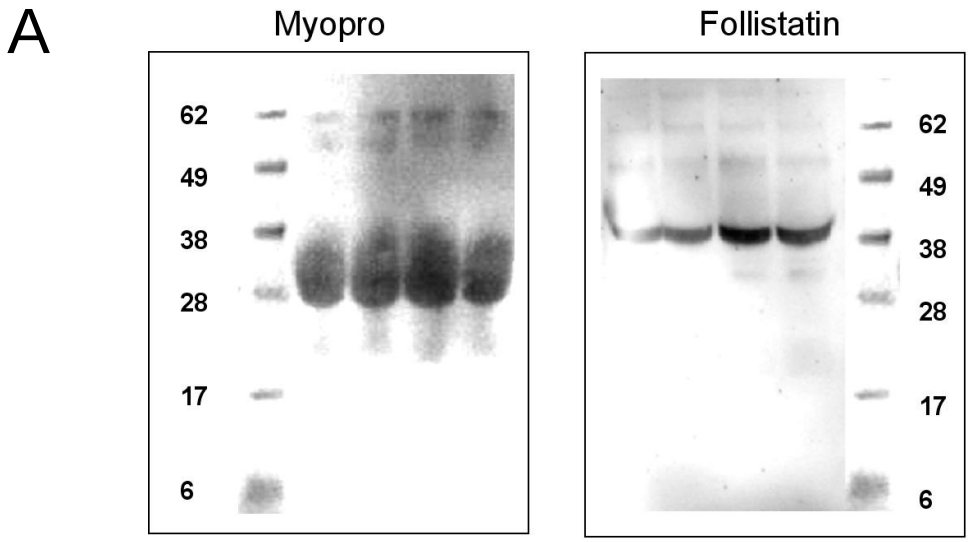

A
Real-time
PCR
Imperacer®
conjugate
target
follistatin
capture
antibody
B
Sample
Polyplex analysis of
different antigens
12 µl sample material
(for each antigen)
2x (double determination):
Add 24 µl detection conjugate mix,
and 6 µl sample
to capture-antibody coated wells
(prepared in advance, ready-to-use)
One-step
incubation
Incubate 45 min/RT
Wash,
add amplification master mix
(including real-time probe)
start amplification in cycler
Taq
real-time PCR (90 min)
Analyze data

Fig.1

Figure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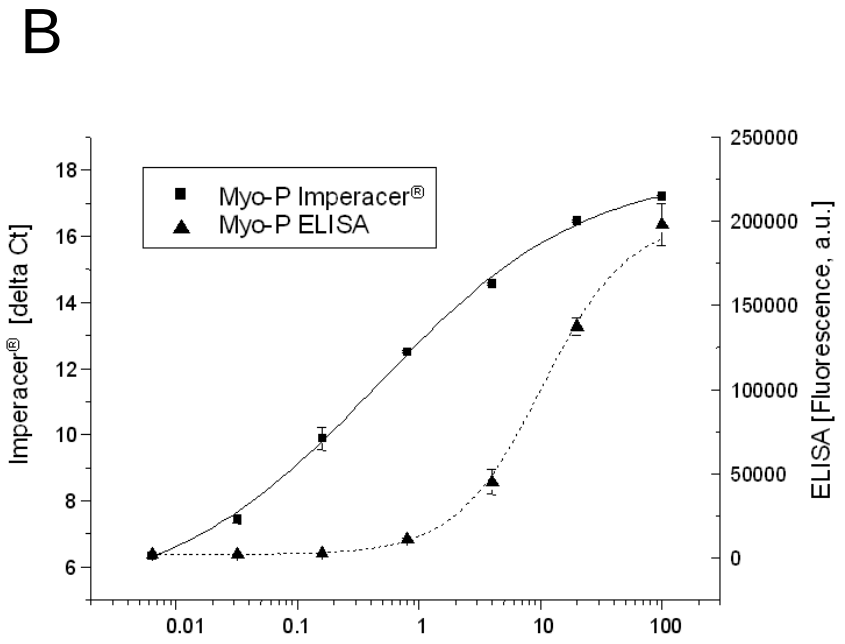
Myopro
Follistatin
62
49
38
28
17
6
62
49
38
28
17
6


B
Myo-P Imperacer®
Myo-P ELISA
Imperacer® [delta Ct]
ELISA [Fluorescence, a.u.]
18
16
14
12
10
8
6
250000
200000
150000
100000
50000
0
0.01
0.1
1
10
100

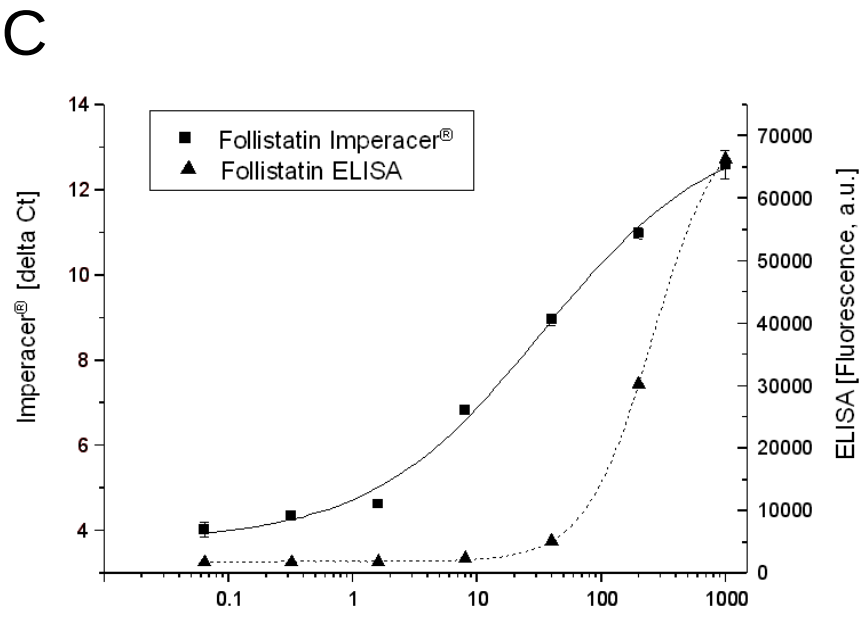

C
Follistatin Imperacer®
Follistatin ELISA
Imperacer® [delta Ct]
ELISA [Fluorescence, a.u.]
14
12
10
8
6
4
70000
60000
50000
40000
30000
20000
10000
0
0.1
1
10
100
1000

Fig.2

Figure

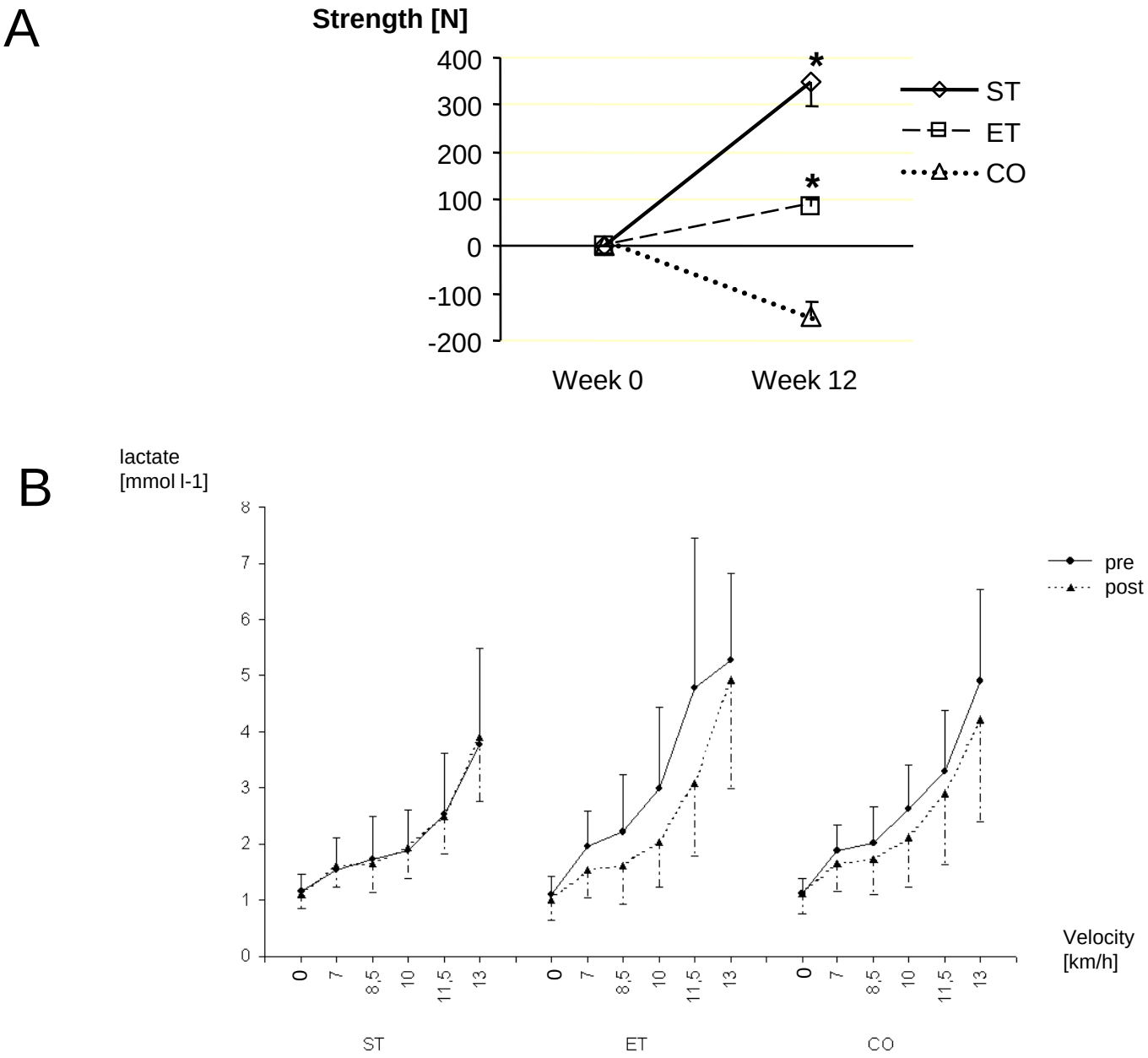
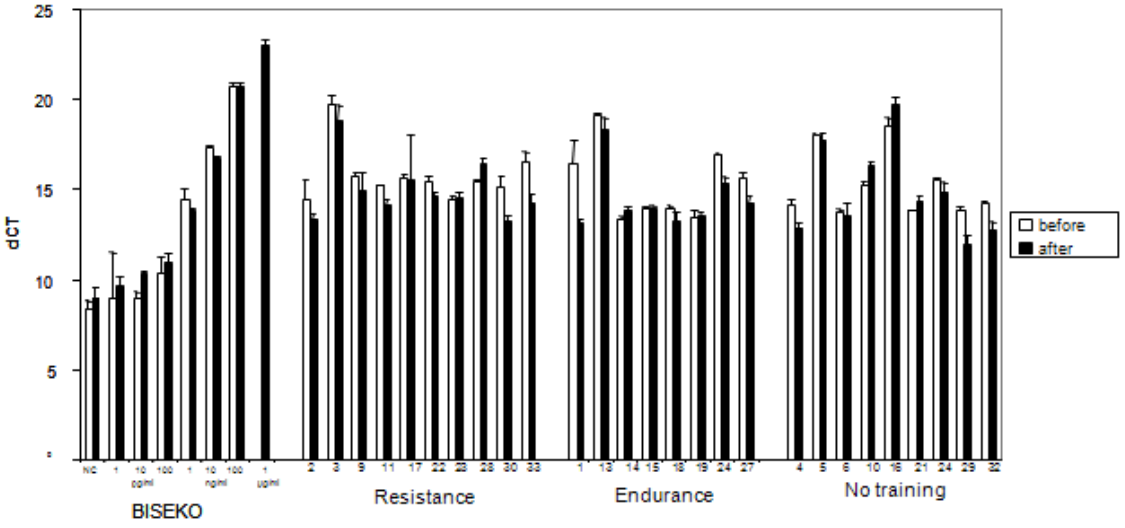

A
Strength [N]
400
300
200
100
0
-100
-200
*
*
ST
ET
CO
Week 0
Week 12
B
lactate
[mmol l-1]
8
7
6
5
4
3
2
1
0
pre
post
0
7
8,5
10
11,5
13
ST
ET
CO
Velocity
[km/h]

Fig.3

Figure

A



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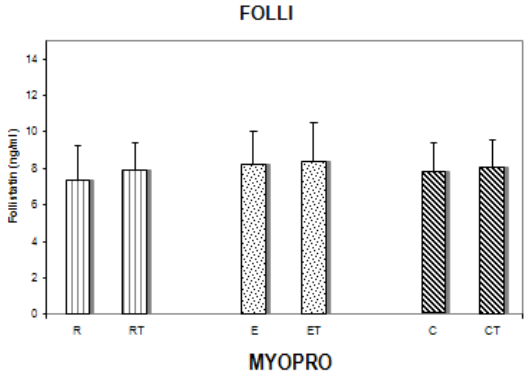

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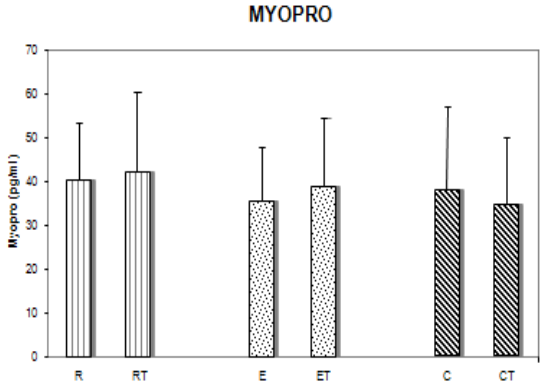

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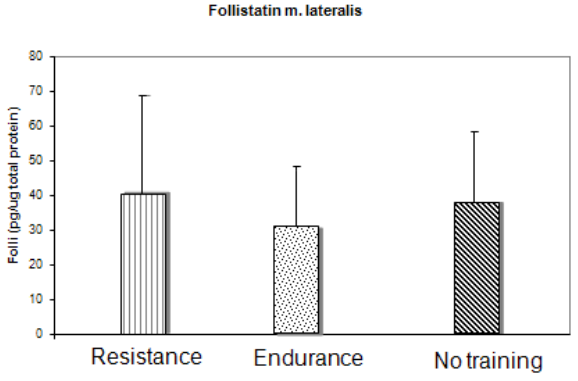


Fig.4

Figure

A

	MYO (ng/ml)	MYOPRO (pg/ml)	FOLLI (ng/ml)
T ETRA	26,00± 4,47	30,66±20	5,48±1,2
BODY	30,33 ± 8,21	81,44±14 *	7,41±1,8
CONTROL	ND	34,66±15	7,70±1,6

B

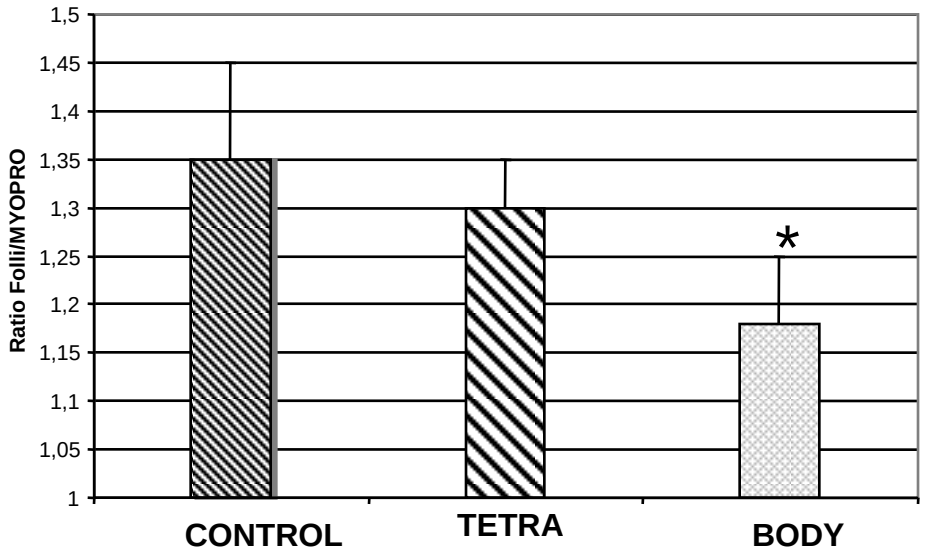
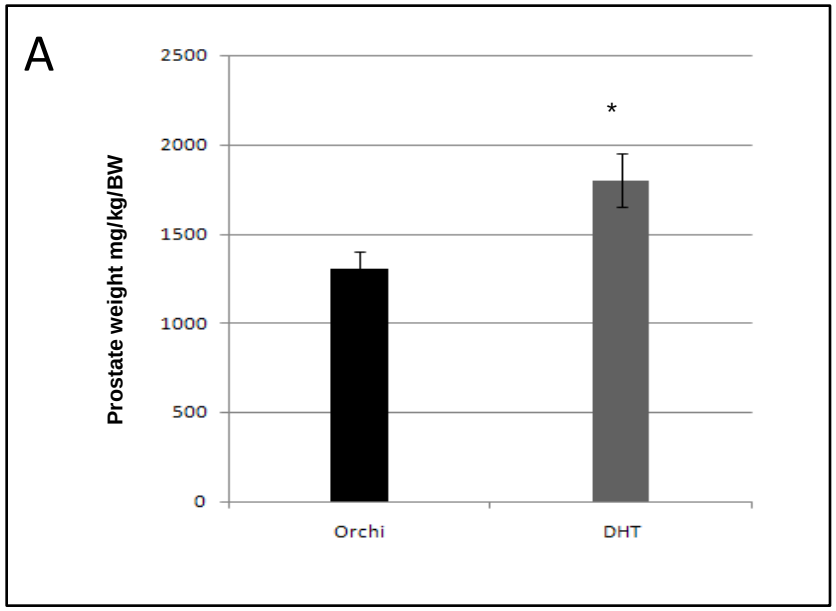
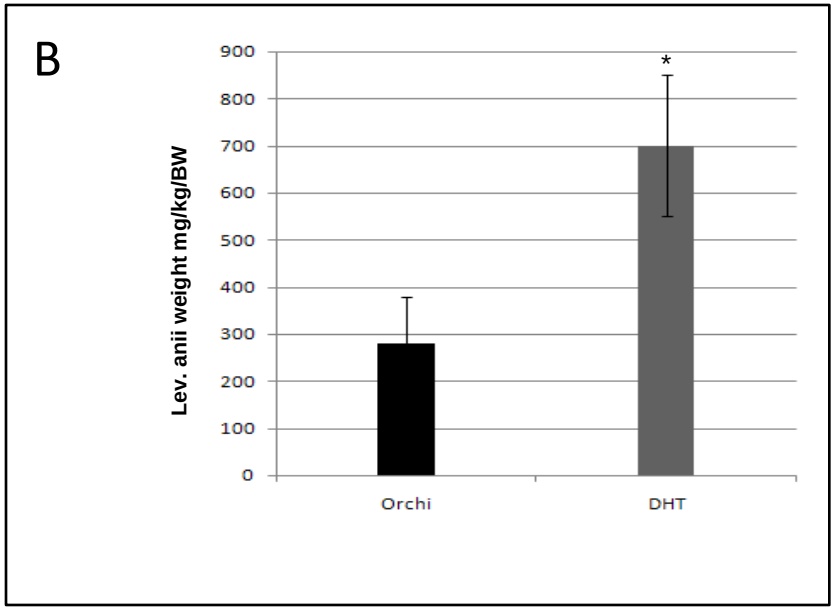
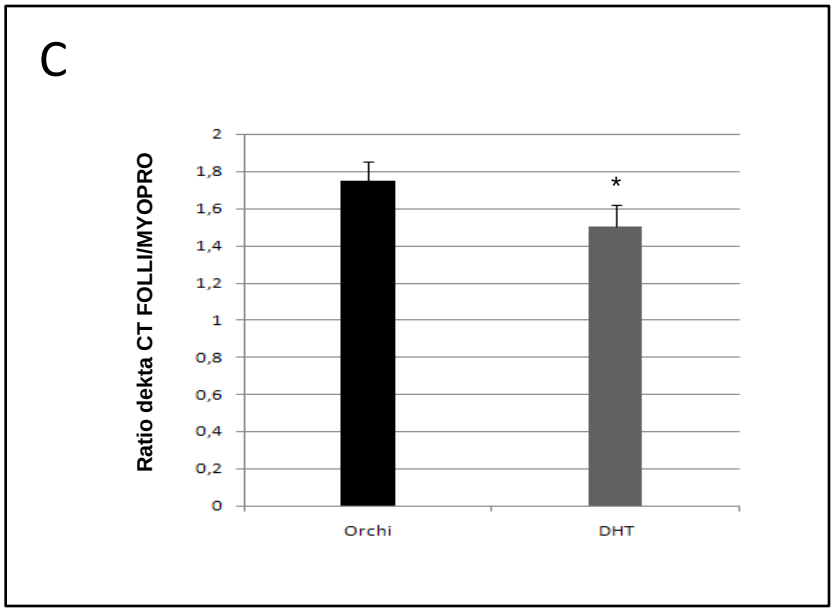


Fig. 5

Figure


A
Prostate weight mg/kg/BW
2500
2000
1500
1000
500
0
*
Orchi
DHT


B
Lev. anii weight mg/kg/BW
900
800
700
600
500
400
300
200
100
0
*
Orchi
DHT


C
Ratio dekta CT FOLLI/MYOPRO
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
Orchi
DHT

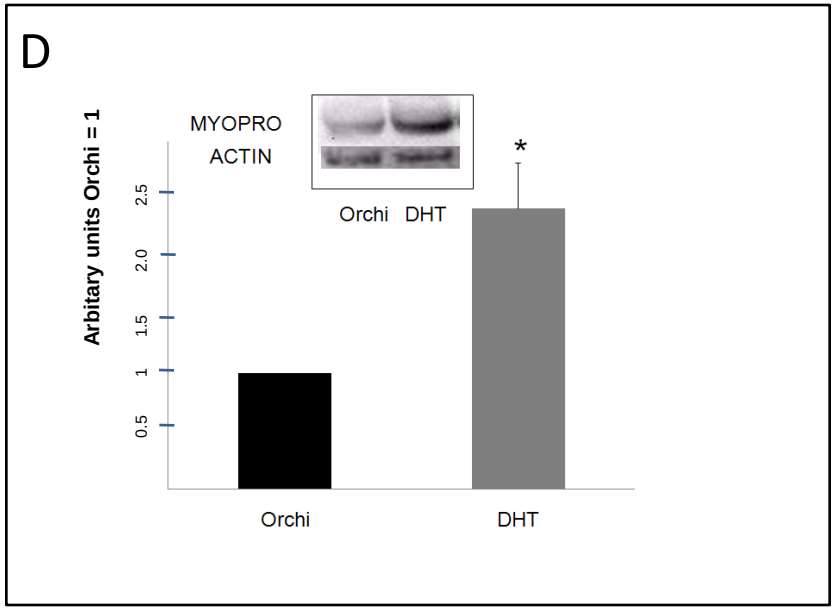

D
Arbitary units Orchi = 1
MYOPRO
ACTIN
Orchi DHT
0.5
1
1.5
2.0
2.5
*
Orchi
DHT

Fig.6