Journal of Steroid Biochemistry & Molecular Biology 165 (2017) 382–395

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Genomic and lipidomic actions of nandrolone on detached rotator cuff muscle in sheep

Martin Flück, PhD[a,*,1], Severin Ruoss[a,1], Christoph B. Möhl[a], Paola Valdivieso[a], Mario C. Benn[b], Brigitte von Rechenberg[b], Endre Laczko[c], Junmin Hu[c], Karl Wieser[d], Dominik C. Meyer[d], Christian Gerber[d]

[a] Laboratory for Muscle Plasticity, Department of Orthopedics, University of Zurich, Balgrist Campus, Zurich, Switzerland
[b] Musculoskeletal Research Unit, Center for Applied Biotechnology and Molecular Medicine, Equine Department, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland
[c] Functional Genomics Center Zurich (FGCZ), ETH and University of Zurich, Switzerland
[d] Balgrist University Hospital, Department of Orthopedics, Zurich, Switzerland

ARTICLE INFO

Article history:
Received 22 April 2016
Received in revised form 5 August 2016
Accepted 8 August 2016
Available online 12 August 2016

Keywords:
Rotator cuff muscle
Adipocyte
Fat
Gene expression
Tenotomy
Anabolic steroids
Androgen receptor

ABSTRACT

Reversal of fatty infiltration of pennate rotator cuff muscle after tendon release is hitherto impossible. The administration of nandrolone starting at the time of tendon release prevents the increase in fat content, but does not revert established fatty infiltration.

We hypothesised that tendon release and myotendinous retraction cause alterations in lipid related gene expression leading to fatty muscle infiltration, which can be suppressed by nandrolone through its genomic actions if applied immediately after tendon release.

The effects of infraspinatus tendon release and subsequent tendon repair at 16 weeks were studied in six Swiss Alpine sheep. In the interventional groups, 150 mg nandrolone was administered weekly after tendon release until sacrifice (N22W, n = 6) or starting at the time of repair (N6W, n = 6). Infraspinatus volume, composition, expressed transcripts, lipids, and selected proteins were analyzed at baseline, 16 and 22 weeks.

Tendon release reduced infraspinatus volume by 22% and increased fat content from 11% to 38%. These changes were not affected by repair. Fatty infiltration was associated with up-regulation of 227 lipid species, and increased levels of the adipocyte differentiation marker PPARG2 (peroxisome proliferator-activated receptor gamma 2). Nandrolone abrogated lipid accumulation, halved the loss in fiber area percentage, and up-regulated androgen receptor levels and transcript expression in the N22W but not the N6W group.

The results document that nandrolone mitigates muscle-to-fat transformation after tendon release via a general down-regulation of lipid accumulation concomitantly with up-regulated expression of its nuclear receptor and downstream transcripts in skeletal muscle. Reduced responsiveness of retracted muscle to nandrolone as observed in the N6W group is reflected by a down-regulated transcript response.

1. Introduction

1.1. Degeneration and repair of the ruptured rotator cuff muscle

Tears of rotator cuff tendons affect a considerable portion of the elderly population [1,2]. Tendon tears lead to musculotendinous retraction, which is associated with muscle transformation by a progressive increase in fat and connective tissue content at the expense of contractile material (fatty infiltration; [3–5]). Even if surgical repair is successful, this transformation of muscle structure is hitherto irreversible (reviewed in [4]) and thereby interferes with restoration of muscle function. The infiltration of muscle with fat tissue is such a risk factor for the success of tendon repair, that an increase in fat content close to, or above 50% of total

* Corresponding author at: Laboratory for Muscle Plasticity, Department of Orthopedics, University of Zurich, Balgrist Campus, Forchstrasse 340, 8008 Zurich, Switzerland.
E-mail address: mflueck@research.balgrist.ch (M. Flück).
[1] Equal contribution as first author.

http://dx.doi.org/10.1016/j.jsbmb.2016.08.005

muscle volume is considered a contra-indication for surgical repair [6]. Muscular changes may render successful reattachment of the ruptured tendon impossible (reviewed in Ref. [4]), due to shortening and stiffening of the detached and atrophic muscle. Thus the prevention of muscular transformation in the retracted muscle is a high priority to allow successful repair of the musculotendinous unit after tendon tear.

1.2. Anabolic steroids prevent fat accumulation and increase lean mass

Anabolic steroids are a proposed treatment against the accumulation of fat and associated muscle atrophy [5] because they increase lean over fat mass by promoting muscle anabolism and reducing fat content [7–9]. We recently reported that the anabolic steroid nandrolone decanoate can prevent the increase in fat content, but does not mitigate atrophy of rotator cuff muscle in sheep and rabbit models if administered at the time of tendon release [10,11]. Anabolic steroids affect a number of systemic and local aspects of lipid metabolism, which may be implicated in fat accumulation in tenotomized rotator cuff muscle. Anabolic steroids reduce lipogenic activity in the liver [12] and enhance lipolytic activity in adipose tissue [13]. Furthermore, anabolic steroids inhibit adipocyte differentiation and affect skeletal muscle directly through genomic effects on transcript expression being associated with cell growth and proliferation, connective tissue development and function as shown in rat experiments [13–17]. Tenotomy has been demonstrated to increase phospholipid and triglyceride content in rat skeletal muscle, which has been postulated to involve the infiltration and expansion of adipogenic cells, as well as de-differentiation of muscle-associated stem cells or fibroblast into adipocytes [18–22]. This raises the possibility that nandrolone prevents fat accumulation in tenotomized muscle [10,11] by multiple mechanisms in skeletal muscle including the abrogation of phospholipid and triglyceride synthesis, the enhancement of lipid oxidation [23,24], through effects on adipocyte differentiation and/or expression of adipogenic pathways in skeletal muscle.

1.3. Ruptured rotator cuff muscle looses responsiveness to nandrolone

Interestingly, nandrolone does not influence fat accumulation in rotator cuff muscle in sheep and rabbit models of tendon release if administered after muscle degeneration has established [10,11]. This biological fact imposes possible limitations for full improvement of muscle function if repair of the tendon is delayed [25,26]. The main cytoplasmic receptor of anabolic steroids, the androgen receptor [17,27], is abundantly expressed in skeletal muscle. In the rat androgen receptor expression is sensitive to muscle loading and also to circulating hormone levels, i.e. being reduced with unloading and increased with nandrolone [28,29]. This suggests that the loss in responsiveness of rotator cuff muscle to anabolic steroids after tendon tear in sheep [30] involves changes in androgen receptor-mediated gene regulation.

1.4. Aim and hypotheses

The purpose of this study was to investigate 1) whether a muscle-based molecular mechanism underlies the increased fat content in sheep infraspinatus muscle 16 weeks after tendon release and 2) whether expression changes explain its mitigation when nandrolone is administered immediately after tendon release before fatty infiltration is established. As metabolism related targets of nandrolone action in skeletal muscle are not established, we first carried out a global assessment of lipid and transcript expression changes with tendon release and repair. For this purpose, we assessed to which extent alterations in transcript expression in detached infraspinatus muscle are suppressed by weekly injections of nandrolone, and whether the transcript response differs when nandrolone is administered immediately after tendon release or only after repair, and whether the response is related to androgen receptor levels. We specifically hypothesised that tendon release enhances transcript expression for gene ontologies (GOs) regulating adipocyte differentiation and lipid metabolism and associated lipid species in detached infraspinatus muscle, and that nandrolone down-regulates this expression program.

2. Materials and method

2.1. Experimental design

Eighteen female Swiss Alpine sheep (two years old; provider Staffelegg, Küttigen (AG), Switzerland) were subjected to release of the infraspinatus tendon in three interventional groups of six animals each (CONTROL: 45.3 ± 1.9 kg, N22W: 47.1 ± 0.8 kg, N6W: 48.4 ± 1.5 kg). N22W received weekly injections of 150 mg nandrolone decanoate (Deca-Durabolin®) into the gluteus maximus muscle in the post-operative phase starting immediately after tendon release. N6W received weekly intramuscular injections of 150 mg nandrolone decanoate starting at the time of tendon repair, i.e. after 16 weeks. At 16 weeks, repair was performed in all groups by a single stage repair of the tendon to the greater tuberosity. Prior to tendon release (PRE), 16 weeks after tendon release (TR) and 6 weeks after repair (END) measurements were made on the operated muscle to characterize retraction, volume and fat content of the infraspinatus muscle. The measures were compared to contralateral control muscle at the end of the experiment (END-CC). Further, biopsy samples from the infraspinatus muscle were collected. Biopsies were subjected to the characterization of muscle composition and transcript expression. Absolute values for the volumetric and cellular adjustments of infraspinatus muscle have in part been reported. They are now included as percentage change to allow the interpretation of the new measurements on molecular parameters with tendon release and repair. The experiment was approved by the local federal authorities (Veterinary Office of the Canton of Zurich, application number 72/2013).

2.2. Tendon release and reattachment

All procedures were carried out on the right shoulder essentially as described [4,30]. The tendon was released by osteotomy of the greater tuberosity ($20 \times 10 \times 10$ mm) using an oscillating saw. The tendon stump was grasped via its attached bone chip through 2 figure-of-8 stitches using Fiberwire USP No. 2 sutures (Arthrex, Inc, Naples, Florida) that passed within a 1.8-mm drill-hole in the bone chip. Subsequently, the tendon and bone chip, with the tied sutures, were wrapped in a silicon tube (Silicone Penrose drain tube, 12 mm diameter; Fortune Medical Instrument, Taipei, Taiwan) to prevent reattachment through scar tissue. Sixteen weeks later, rotator cuff repair was performed. The bone chip was reattached to its original site, or as near as possible by connecting the remaining sutures to a 3.5-mm self-tapping cortical bone screw (Synthes, Paoli, USA) with a washer. During the first three weeks of the rehabilitation period, stress to the repaired tendon was eased by reducing full weight bearing through suspension of the sheep in a loose belt and by attaching a ball to the claws. After 6 weeks of reattachment, the animals were sacrificed.

2.3. Muscle anatomy

Immediately after each surgical intervention, magnetic resonance imaging (MRI) and computed tomography of both shoulders

was performed with the animal still under general anesthesia. MRI scans were evaluated for bone chip retraction, muscle volume using a Digital Imaging and Communications in Medicine (DICOM) viewer (OsiriX v.5.6 32-bit) as described [11].

2.4. Sampling of muscle tissue

Biopsies were harvested during each of the surgical interventions from the middle portion of the lateral compartment of the operated infraspinatus muscle. This was achieved via Bergstroem biopsy needles with a 5-mm diameter (Dixons Surgical Instruments LTD, Wickford, UK) through a 15-cm curved incision 2 cm caudal to the scapular spine. Biopsy samples were rapidly dissected free of excessive connective tissue and blood, frozen in melting isopentane and kept individually in sealed 2-mL cryotubes (Greiner, Frickenhausen, Germany) at −80 °C until use. At the time point of sacrifice, the entire infraspinatus muscle and its contralateral control, were excised under general anesthesia and frozen at −80 °C.

2.5. Characterization of muscle composition (morphometry)

Immunohistochemical detection and morphometric quantification of muscle fibers, lipid droplets and extracellular matrix was carried out essentially as described using cryosections [11,31]. In brief, cryosections were prepared at 12-μm thickness perpendicularly to the major axis of muscle fibers within the biopsy. Sections were stored at −80 °C until being processed using the Goldner trichome method to distinguish contractile from connective tissue, and Red Oil O staining to detect lipid. The stained sections were digitally recorded using an IX50 microscope with a digital camera DP72 (Olympus Schweiz AG) that was operated with the Cell Sens Dimension software (Version 1.6, Olympus Corporation). Images were exported as tiff-file into Adobe Photoshop CC (Adobe Systems Incorporated) or ImageJ software (http://rsbweb.nih.gov/ij/). Goldner trichome stained sections were analyzed using the lasso tool for the area percentage of muscle fibers (red structures) and connective tissue (green and non-stained structures). Lipid content was assessed in Red Oil O stained sections from the red signal using a macro with imageJ software (http://rsbweb.nih.gov/ij/). Numbers from the different fields were summed for the respective cellular structures to calculate the respective area percentages of muscle fibers, lipid and extracellular matrix for the entire cross section/biopsy. The percentage of extracellular matrix was calculated as the difference between the respective area percentage of connective tissue (as estimated from the Goldner staining) and lipid (as estimated in the Red Oil O staining).

2.6. Lipidome analysis

Lipid species were analyzed in infraspinatus muscle samples using ultrahigh performance liquid chromatography–tandem mass spectrometry coupled by nano electrospray source ionization (UPLC–nanoESI–MSMS) based on established protocols [6,32] and SOPs for lipid analysis of the FGCZ. Three biological replicas from the excised muscle of the operated side (sample END) and its contralateral control (sample END-CC) from the CONTROL, N6W and N22W group 6 weeks after repair were analyzed. In brief, 5 mg of muscle tissue was cryosectioned, compounds extracted in cold MetOH: MTBE: H20 = 360: 1200: 348 using a full glass Potter type homogenisator and 10ul of LysoPC (17:0; Avanti Polar Lipids, Inc. Sigma) and ^{13}C-Sorbitol (Sigma) was added as internal standards. The upper (non-polar) phase was recovered after a centrifugation step (5 min at 1000g, at 4 °C), collected and stored at −30 °C. All extraction steps were carried out in dichlormethane-rinsed Duran glassware using MS-Grade compounds (CHROMOSOLV®, Sigma). Prior to analysis the lipid extracts were dried down under a steam of nitrogen and reconstituted in 100uL of 10 mM ammonium acetate in 80% aqueous methanol.

Lipid compounds were separated on nanoAquity UPLC (Waters) equipped with a C18 reversed phase column (HSS T3 1.7 μm, 0.2 mm x 150 mm, Waters) applying a gradient of 5 uM ammoniumacetate in acetonitril–water 40:60 v/v (A) and 5uM ammoniumacetate in acetonitrile – isopropanol 10:90 v/v (B) from 95% A to 2% A. The injection volume was 1 μL and a flow of 3 μL/min was applied. The UPLC was coupled to a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher) by a nanoESI source that was operated in positive polarization mode, applying a voltage of 1.8 kV. The MS was operated in data independent acquisition mode (All Ion Fragmentation) with stepped collision energy setting (CE 30–35–40 V). All samples were injected twice (technical replicates). Mass spectrometry data sets were processed with Progenesis QI (Nonlinear Dynamics, A Waters Company). Compound ions were detected and quantified by a multi-step procedure including the retention time (RT) alignment of observed m/z signals, the construction of a consensus m/z – RT map, grouping of co-eluting adducts and quantification of detected compounds through all samples. Detected compound ions were further annotated by database searches based on derived neutral masses, isotopic patterns and the match of observed fragmentation spectra and theoretical fragmentation spectra. Database searches were run with a tolerance of 50 mD and included LipidMaps (www.lipidmaps.org) and the Human Metabolome Data Base (HMDB, www.hmdb.ca). Compounds, which demonstrated significant differences (see paragraph 'statistics' below), were allocated its respective Pubchem substance identifier (SID). Data sets were deposited in MetaboLights (http://www.ebi.ac.uk/metabolights/about) as study MTBLS322.

2.7. Transcript expression

Total RNA was isolated from three biological replicas for all time points (PRE, TR, END and END-CC) of the CONTROL, N22W and N6W group as described [33] and selected samples were subjected to RNA sequencing using standard protocols. In brief, an approximate of 15 mm3 of cryosections was pooled and processed using the RNeasy kit (Qiagen). The volume of the sectioned tissue was estimated based on the cross-sectional area, as measured with a ruler for representative sections on slides under a microscope, the thickness and the number of pooled sections. The quantity and quality of total RNA were evaluated using Qubit® RNA BR Assay Kit (LifeTechnologies, USA) and Agilent RNA 6000 Nano kit with the Agilent 2100 Bioanalyzer instrument (Agilent Technologies Inc., Santa Clara, USA), respectively. RNA sample was accepted for the study when the RIN was ≥7.0.

Library products were generated from 1 μg total RNA subsequent to the removal of ribosomal RNA with the Epicentre Ribo-Zero™ Magnetic Gold Kit and fragmentation of the enriched RNA using the Illumina TruSeq® Stranded total RNA protocol. Subsequently first strand cDNA synthesis was carried out using random hexamers, followed by removal of the RNA template and second strand cDNA synthesis in the presence of dUTP in place of dTTP. Next, the 3′ ends of the blunt fragments were adenylated, multiple indexing adapters ligated, the cDNA amplified using PCR and the quantity and quality of generated libraries determined by qPCR with Illumina adapter specific primers using the Roche LightCycler® system (Roche Diagnostics, Basel, Switzerland) and an Agilent Technologies 2100 Bioanalyzer with a DNA-specific chip, respectively. Diluted libraries (10 nM) were pooled, used for cluster generation according to the manufacturer's recommendations using TruSeq SR Cluster Kit v3-cBot-HS reagents and sequenced

with TruSeq SBS Kit v3-HS reagents on Illumina HiSeq 2500 in the high output mode. On average 50′000′000 reads were sequenced per library. Raw reads were cleaned by removing adapter sequences and by trimming the first three and last four bases. The resulting high-quality reads were aligned to the current genome build (Oar_v3.1.75, Ensembl) using STAR (v2.3.0e) [34]. The resulting mapping rate was 90%. Approximately 55% of the reads corresponded to mRNA. Counting was performed using the

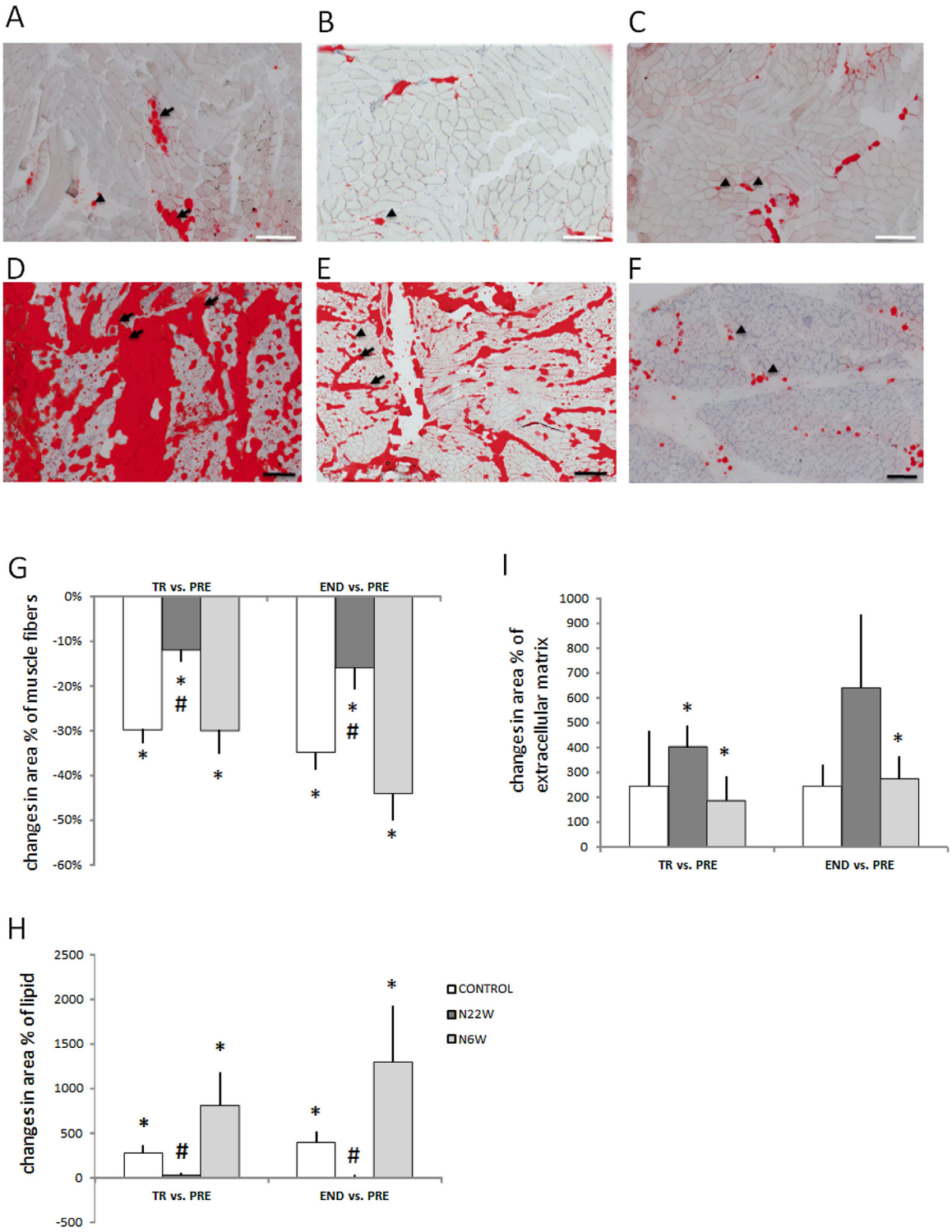

Fig. 1. Changes in muscle compartments with tendon release and repair. A–F) Representative images of Red Oil O-stained cross sections of biopsies from infraspinatus muscle of sheep collected before (A–C) and after tendon release and 6 weeks of repair (D–F) for the intervention group without (CONTROL; A, D) and with nandrolone administration for 6 weeks (N6W; B, E) or 22 weeks (N22W; C, F). Arrows and arrowheads point to respective examples of individual adipocytes and groups of Red Oil O-positive adipocytes, respectively. White and black scale bars correspond to 200 and 500 micrometers, respectively. G-I) Bar graphs showing mean + standard error (SE) of the changes in the area percentage of the muscle fibers (G), fat (H) and extracellular matrix (I) compartment of infraspinatus muscle, 16 weeks after tendon release (TR. vs. PRE) and 6 weeks after repair (END vs. TR). *, $p < 0.05$ vs. PRE. #, $p < 0.05$ vs. CONTROL. Repeated-measures ANOVA with post hoc test of Fisher. N = 6 per group.

countOverlaps-method from the IRanges package in Bioconductor (v2.13 with R 3.0.2). Raw signals were expressed as 'normalized signals', reflecting the frequency of the individual gene transcripts. Subsequently the 'relative concentration' of gene transcripts was estimated based on the product of multiplying the 'normalized value' for each transcript with the measured 'RNA concentration' per volume of muscle tissue. Data sets were deposited under series record GSE80374 in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ezazgomefzupxyf&acc=GSE80374).

2.8. Characterization of protein expression

Relative concentrations of proteins of interest per total protein were assessed essentially as described [35]. In brief, 10 μg total proteins in total homogenate from the different sample points for either intervention, for one sheep each, were loaded in adjacent lanes aside a common sheep muscle sample and a prestained molecular weight marker (#161-0374, Biorad) on one 7.5% SDS-PAGE using precast gels (Biorad Mini-protean TGX Stain-free). Proteins were separated, western blotted on nitrocellulose membrane (Schleicher & Schuell, GE Healthcare) and proteins of interest were detected by incubating the membrane with specific antibodies in 5%-milk/1% BSA –TTBS and enhanced chemoluminescence.

PPARG isoforms 1 and 2 were detected using 1: 1′000 dilution of monoclonal mouse antibody (no. LS-C178333 from LS Bio, Lifespan Biosciences Inc., LabForce AG, Nunnigen, Switzerland) and 1: 20′000 dilution of horse-radish peroxidase-conjugated secondary antibody (goat anti-mouse, #9917 Sigma). Androgen receptor was detected using a 1:250 dilution of rabbit pAb PG-21 (#06-680, Merck Millipore, Zug, Switzerland), respectively, and a 1:20′000 dilution of horse-radish peroxidase-conjugated secondary antibody against rabbit immunoglobulins (#55676, MP Biomedicals, Basel, Switzerland). The respective signals were recorded using a PXi System (Syngene, Labgene Scientific SA, Chatel St Denis, Switzerland). Recorded signal was quantified using Quantity one (Biorad, Cressier, Switzerland) with the rectangular mode. Signal was scaled relative to the common sample that was present on all blots and subsequently normalized to the mean of pooled pre-values for each blot.

2.9. Statistics

Statistical tests for muscle composition and protein expression were carried out using SPSS (version 22, IBM corporation). Effects on the operated muscle side were assessed with a repeated ANOVA for the factor 'intervention group' (CONTROL, N22W, N6W) and the repeated factor 'time point' (PRE, TR, END). Data were also compared to the results in the END-CC as revealed by a two-factorial ANOVA for the factors 'muscle side' (operated, contralateral) x 'intervention group' (CONTROL, N22W, N6W). A post hoc test for the least significant difference was deemed appropriate for localizing effects. Results were displayed using Prism (Version 5.01, GraphPad Software, Inc.). Linear relationships were assessed using Pearson correlations. A p-value < 0.05 was called significant.

'Relative concentrations' of gene transcripts were assessed using permutations of T-tests with the Significance Analysis of Microarrays test (SAM) running as an applet in Microsoft Excel software [36]. Affected gene transcripts were identified from a comparison of paired classes at a false discovery rate (FDR) of 2%, extracted using a manual process in MS-Excel (Microsoft, Kildare, Ireland) and subsequently analyzed for affected GOs with MetaCore software (GeneGo, Thompson Reuters) using the annotation from homologue human genes. GOs were considered significantly enriched if the *p*-value of enrichment was below the FDR and if the counts of altered transcripts for the respective GO pointed significantly in one direction based on a p-value < 0.01 for a binomial distribution ('Binomdist' function in MS-Excel). Subsequently GOs being associated with the muscle, fat and extracellular compartment were identified and extracted based on whether they contained the mesh terms: 'muscle', 'skeletal muscle' or 'fiber'; 'lipid', 'fat'; 'extracellular', 'matrix', 'connective tissue' or 'adhesion'. In case of overlap, only the parent GO was retained.

For the assessment of ion abundance, the mass spectrometry data set was limited to those compounds being detected in all analyzed samples. The total lipid signal was defined as the summed abundance of all ions being detected in one given sample. For each sample, raw signals of each compound were related to the internal LysoPC (17:0) standard. Changes were assessed based on permutations of T-tests using SAM [36]. A paired class design was applied to identify differences between the operated muscle and its contralateral control. An unpaired class design was applied to

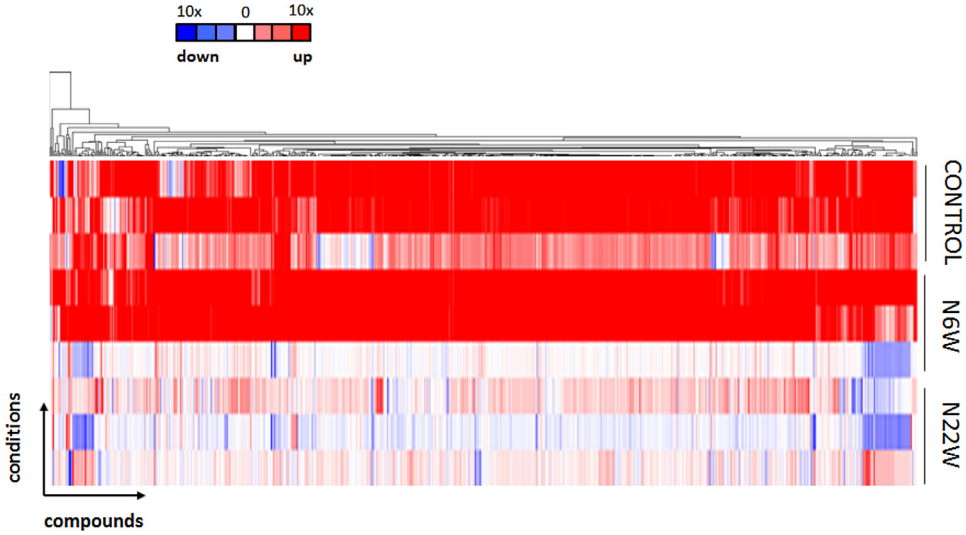

Fig. 2. Muscle lipidome after tendon release and repair. Cluster of the fold differences for 703 detected lipid compounds between the contralateral and operated side of infraspinatus muscle for the CONTROL, N6W and N22W group after 16 weeks tendon release and 6 weeks of repair. Three biological replicas were assessed per condition. Lipid level alterations are given in color-coding (up: red; down: blue) and are ordered according to the correlation of their response in clusters as indicated in the dendrogram to the left.

identify changes between the intervention groups for post vs. contralateral differences. A false discovery rate of 5% was deemed significant. Output was exported into MS-Excel for table assembly. For the altered species, the corresponding lipid class was manually extracted by consulting the Pubchem entry. Lipid classes were considered significantly enriched if the number of altered lipid species of the respective class pointed in one direction based on a p-value < 0.01 (Binomdistribution). Affected lipid compounds and transcripts were subjected to correlation analysis using the expression correlation network plugging of the cytoscape software (2.5.0) at a condition of r > |0.99|.

3. Results

3.1. Anatomic alterations

The changes after 16 weeks of tendon release in the CONTROL group included a myotendinous retraction of 5.8 + 0.5 cm and a 22% loss of muscle volume. The area percentage of muscle fibers was reduced by 30% while the area percentage of fat was increased by 281% (Fig. 1), reaching together with the extracellular matrix a value corresponding to 38% of total area percentage of the muscle. Muscle alterations with tendon release were not substantially altered in the 6 weeks after repair.

Compared to CONTROL, nandrolone administration starting immediately after tendon release (N22W) prevented the increase in area percentage of fat (23% vs. 277% and −1% vs. 398%, respectively) and mitigated the reduction in area percentage of muscle fibers after tendon release (12% vs. 30% and 16% vs. 35%, respectively), as well as repair (Fig. 1). The area percentage of the extracellular matrix was increased by 368% after 16 weeks of tendon release under nandrolone administration and tended to be different from the not-significantly affected values in the CONTROL group (p = 0.06). The administration of nandrolone for 6 weeks starting with tendon repair (N6W) did not affect the changes in muscle volume (−23% vs. −31%) and muscle composition (muscle fiber area: −44% vs. −35%; lipid: 1260& vs. 398%, extracellular matrix: 260% vs. 240%) as seen in the CONTROL group with tendon release and repair.

3.2. Metabolic alterations

703 lipid species were detected in the infraspinatus muscle (Fig. 2). The pool sizes of 227 lipid species in the CONTROL group were increased after tendon release and 6 weeks of repair compared with the non-operated contralateral control. The changes concerned the lipid class of fatty acyls, phospholipids, prenol lipids, polyketides, sterol lipids and sphingolipids but not glycolipids (Table 1).

Table 2
Nandrolone-dependent non-polar compounds.
Number, median fold changes and the *p*-value for lipid classes in sheep infraspinatus muscle 16 weeks after tendon release and 6 weeks after repair between the N22W and CONTROL group (n = 3 per group). Values were calculated from differences versus contralateral controls in the respective group.

N22W			
lipid class	*count*	*median change*	*p-value*
fatty acyl	117	0.003	6.0E−36
glycerolipid	1	0.005	5.0E−01
phospholipid	42	0.001	2.3E−13
polyketide	25	0.003	3.0E−08
prenol lipid	33	0.004	1.2E−10
sphingolipid	9	0.001	2.0E−03
sterol lipid	16	0.003	1.5E−05

In the N6W group, 185 of the 227 lipid species in the CONTROL group were increased after tendon release and repair compared with the contralateral control (Table 1). In the N22W group, the administration of nandrolone starting immediately after tendon release mitigated the increase of lipid species in the operated muscle (Table 2, Fig. 2). In consequence, in this group no lipid species was increased after tendon release and repair.

3.3. Expression of androgen receptor and the adipocyte differentiation marker PPARG

Protein levels of isoform 2 (PPARG2) but not isoform 1 (PPARG1) were increased 16 weeks after tendon release, in all the three intervention groups (Fig. 3A–C). Neither of the PPARG isoforms was affected by nandrolone.

Mean protein levels of the androgen receptor were not affected 16 weeks after tendon release in the CONTROL group but increased 5-fold 6 weeks after repair (Fig. 3D/E). Androgen receptor levels were increased 6-fold after tendon release under nandrolone administration in the N22W group and remained at an elevated level after repair. In the N6W group, nandrolone administration after repair increased androgen receptor levels above the level alterations seen after 16 weeks of tendon release but this did not differ to the effects seen in the CONTROL group.

3.4. RNA concentration with tendon release, repair and nandrolone administration

RNA concentration per muscle volume was not altered 16 weeks after tendon release, or 6 weeks after repair in the CONTROL group (Fig. 4). Nandrolone administration starting after tendon release (in the N22W group) increased RNA concentration by 67% at tendon repair (Fig. 4) before RNA concentration decreased below pre-levels 6 weeks after repair. Nandrolone administration starting

Table 1
Alterations in lipids after tendon release and repair.
Number of significantly affected lipid species in the non-polar fraction (SAM, FDR = 5%), median fold changes per lipid class and the *p*-value for the change over the entire lipid class, respectively, in sheep infraspinatus muscle 16 weeks after tendon release and 6 weeks after repair (END) versus contralateral controls (END-CC) in the CONTROL and N6W group, respectively (n = 3). Values were calculated based on raw signals of each compound relative to the internal standard LysoPC (17:0).

CONTROL					N6W			
lipid class	count	median change	[min-max]	p-value	count	median	[min-max]	p-value
fatty acyl	115	978.6	[4.3–68470.8]	2.00E−35	93	10.78	[0.1–4516.2]	1.01E−28
glycerolipid	1	1367.5	[1367.5–1367.5]	5.00E−01	1	26.59	[26.6]	5.00E−01
phospholipid	38	103.9	[1.0–13427.5]	4.00E−12	31	4.02	[0.1–839.9]	4.66E−10
polyketide	25	1050.6	[14.6–2731.1]	3.00E−08	19	14.87	[0.6–238.2]	1.91E−06
prenol lipid	32	831.6	[15.4–14215.4]	2.00E−10	21	15.35	[0.1–12743.5]	4.77E−07
sphingolipid	10	665.3	[3.9–4226.6]	1.00E−03	7	33.98	[8.2–1028.3]	7.81E−03
sterol lipid	16	452.7	[1.0–30035.9]	2.00E−05	13	27.46	[0.4–18206.0]	1.22E−04

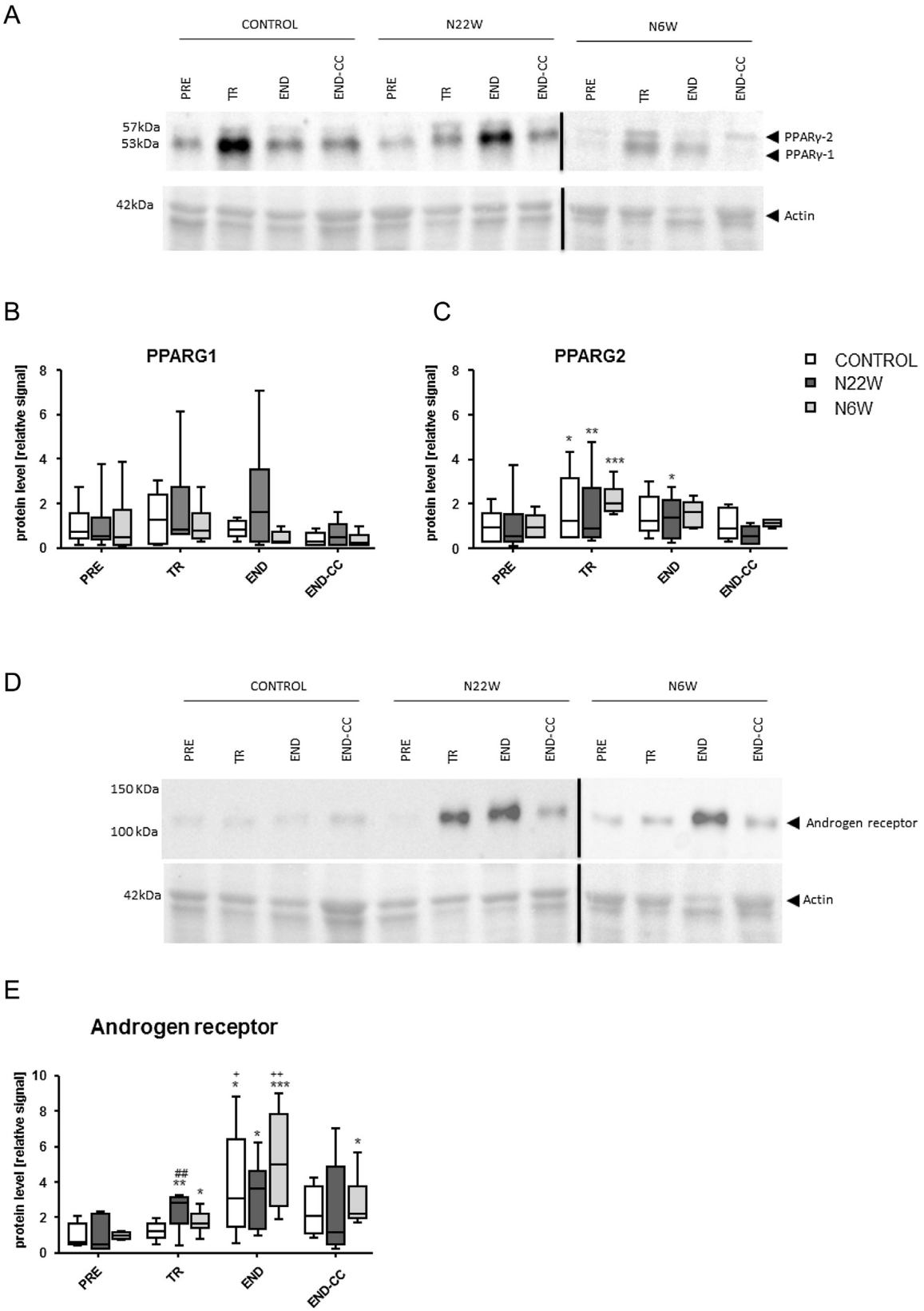

Fig. 3. Protein levels of adipocyte marker PPARG and androgen receptor. A, D) Representative example of immunoblots showing the abundance of PPARG isoforms and androgen receptor in infraspinatus muscle prior to tendon release (PRE), at repair (TR) PRE) and 6 weeks after repair (END) in the CONTROL, N6W and N22W groups. The dividing line illustrates how the records from different blots were assembled. B, C, E) Whisker plots visualizing the median, 25% and 75% confidence interval (central line and borders of box) and minima/maxima (extremes of the lines) of the protein abundance for the PPARG1 and PPARG2 isoforms, and androgen receptor. *$P < 0.05$, **$P < 0.01$ or ***$P < 0.005$ vs. PRE; +$P < 0.05$ or ++$P < 0.01$ vs. TR, #$P < 0.05$ or ##$P < 0.01$ vs. CONTROL. Repeated-measures ANOVA with post-hoc test of Fisher. N = 6 per group.

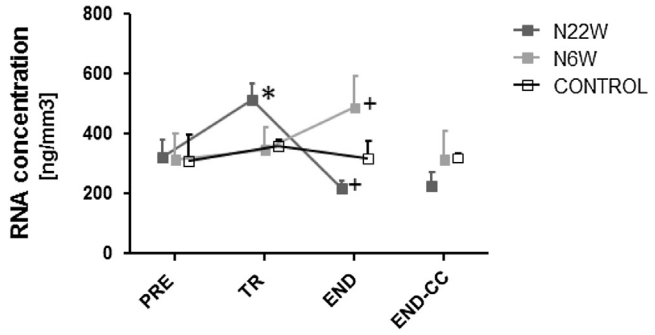

Fig. 4. Ribonucleic acid concentration with tendon release and nandrolone administration. Line graph visualizing the mean + SE of RNA concentration in sheep infraspinatus muscle in the CONTROL, N6W and N22W group. *P < 0.05 vs. PRE; +P < 0.05 vs. TR, #P < 0.05 vs. CONTROL. Repeated-measures ANOVA with post-hoc test of Fisher. N = 3 per group.

after repair (in the N6W group) tended to increase the RNA concentration by 40%.

3.5. Global alterations in transcript expression with tendon release

13821 gene transcripts were expressed in infraspinatus muscle (the transcriptome). In the CONTROL group, tendon release affected the muscle transcriptome in a specific pattern and this was further modified with the subsequent repair (Fig. 5A). The relative concentration of 1397 transcripts was influenced 16 weeks after tendon release: 945 transcripts were down-regulated 16 weeks after tendon release, emphasizing an enriched affection of GOs being associated with muscle, and contrary to our expectation, fat (Fig. 5B, Table 3, Table S1). 452 transcripts were up-regulated after tendon release; being enriched for GOs that are associated with muscle fibers, the extracellular matrix, the vasculature and nerves. The highest significance for enrichment concerned the GO of 'extracellular structure organization' which transcripts were up-regulated (Table S1 and S2). A few transcripts being associated with adipocyte differentiation (i.e. ACSM1, ADIPOQ, FABP4, PLIN1, PPARG2) and two extracellular factors, only, associated with dedifferentiation of fibroblasts (i.e. FAP, DPP; [37], were increased 16 weeks after tendon release but the corresponding GO was not significantly affected.

Tendon repair produced qualitative alterations in the expression pattern of transcripts being associated with contraction, mitochondrial metabolism and protein translation (Fig. 5A). At the selected threshold of statistical significance (FDR of 2%), none of the measured transcripts changed in concentration 6 weeks after repair compared to the situation after tendon release.

3.6. Anabolic steroid stimulates transcript expression

Compared to the response in the CONTROL group, the concentration of 1994 gene transcripts was increased at repair with nandrolone administration (in the N22W group) starting with tendon release. The major theme was the up-regulation of GOs associated with the extracellular matrix and neurogenesis. As well GOs associated with skeletal muscle and the catabolism and biosynthesis of lipids were up-regulated (Fig. 5B; Table S3). The concentration of thirteen transcripts being associated with adipocyte differentiation was increased at repair with nandrolone administration during the 16 weeks after tendon release, but this did not include PPARG2.

Nandrolone administration starting with repair (in the N6W group) modified the response of 10756 gene transcripts; 273 being increased and 10483 being reduced 6 weeks after repair compared to the response of the CONTROL group. This response of the N6W group differed to the response of the N22W group to nandrolone administration during the 16 weeks after tendon release (Fig. 6A). 1722 transcripts, which were increased at repair in the N22W group compared to the CONTROL group, were down-regulated in the N6W group with nandrolone administration during the 6 weeks of repair (Fig. 5A). 272 transcripts were similarly up-regulated in both the N6W and N22W group; the majority of which was associated with neurogenesis (Table S4). All gene ontologies, which reflected the altered gene transcripts, demonstrated altered responsiveness to nandrolone administration in function of whether nandrolone was administered during the 16 weeks after tendon release (N22W group) or only during the 6 weeks after repair (N6W group; Fig. 6B). This included 37 gene ontologies being involved in lipid metabolism, muscle contraction, adhesion and angiogenesis (Table S3).

3.7. Interdependencies

Network analysis of the correlations between fold changes in gene transcripts and lipid compounds over all three interventions identified 3 major entities which described linear relationships within the class of lipid compounds and gene transcripts, but not between them (Fig. 7).

4. Discussion

4.1. Synopsis

The prevention of muscle transformation including fiber atrophy, fat accumulation, and matrix increases is a priority for successful repair of rotator cuff tendons (reviewed in [4,38]). We recently demonstrated that the early administration of nandrolone is a possible measure against the accumulation of intramuscular fat with tendon release of rotator cuff muscle in sheep [11]. The fat mobilizing effect of nandrolone has been explained by alterations in the handling of liver-derived high-density lipoprotein cholesterol [7,39]. To our knowledge, except for altered expression of the lipoprotein apolipoprotein D, it is not known whether nandrolone exerts direct effects on lipid metabolism in skeletal muscle [14,40]. In order to resolve the underlying mechanism, we applied a combination of high-throughput molecular measurements and morphometric analysis in an established sheep model for tendon tears of rotator cuff muscle. The results provide first evidence that nandrolone administration reduces the wasting of muscle fibers (Fig. 1G) and exerts a global influence on transcript and lipid expression in the released infraspinatus muscle. The effect of nandrolone on the transcriptome differed from the alterations when nandrolone was administered after repair and this was related to protein levels of the androgen receptor.

4.2. Limitations

A number of factors may be considered when valuing our data. First, no time point assessed the early adaptations with tendon release and the injection of the anabolic steroid. Therefore possibly important first molecular adjustments of muscle to tendon release, which regulate adipogenesis [41], are missed. However the study of the early responses to tendon release were not an option for the current investigation as the focus was on testing the effect of nandrolone administration on lipid accumulation and confirming the reduced effect of nandrolone administration in retracted muscle. Importantly, the injections of the steroid were provided weekly and prevented the elevation of fat content at the earliest selected time point of measurement, 16 weeks after tendon release after which lipid content only tended to increase during repair (p = 0.11; Fig. 1H). Therefore one would expect that transcript

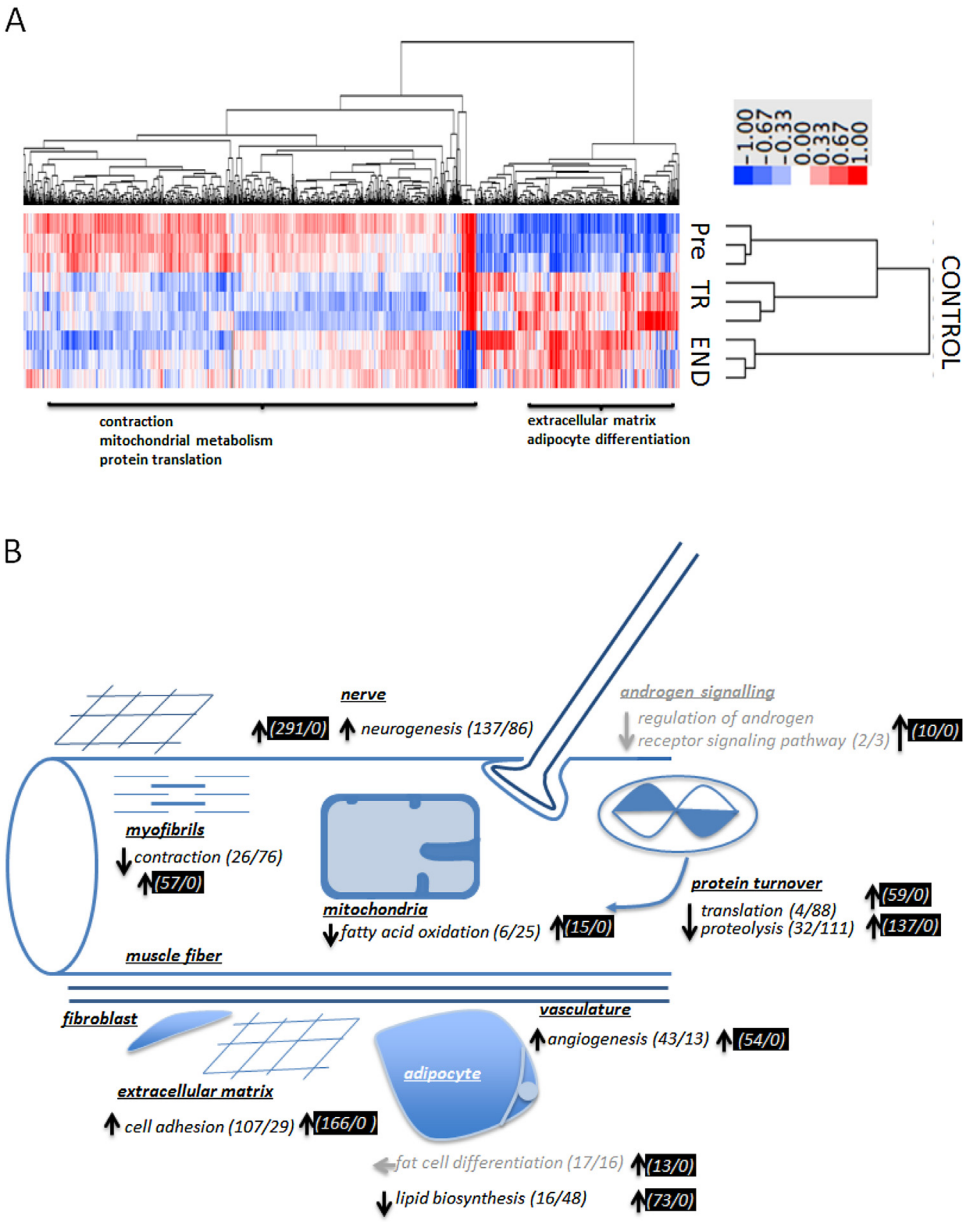

Fig. 5. Profile of muscle transcriptome alterations with tendon release and nandrolone administration. A) Supervised heat map visualizing the concentration of the 1397 altered gene transcripts in infraspinatus muscle of sheep before (PRE), 16 weeks after tendon release at repair (TR) and 6 weeks after repair (END) in the CONTROL group. Transcript data were log-transformed, mean-centered for genes and subjected to centered cluster analysis for genes and arrays. Three biological replicas were assessed per condition. Transcript levels are given in color-coding (up: red; down: blue) and are ordered according to the correlation of their response in clusters as indicated in the dendrogram. Specific gene ontologies of interest are indicated with arrows and brackets. B) Sketch of the main GOs, reflecting muscle processes and compartments, which were affected 16 weeks after tendon release without (CONTROL) or with nandrolone administration. Number in brackets refers to the counts of affected transcripts (increased/decreased) for the respective GO. GOs shown in grey font were not significantly affected in a specific direction. The influence of nandrolone on fold changes with tendon release is indicated in black boxes and arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression of processes, which underlie adipocyte differentiation (or proliferation), lipid biosynthesis and lipid storage, would be specifically lowered in 16 weeks released muscles with nandrolone administration. Secondly, metabolic measurements outside the muscle compartment were not part of our experiment because the focus of our investigation was put on the contribution of local mechanisms to fatty infiltration and atrophy of rotator cuff muscle with tendon release and the altered responsiveness to nandrolone. Nonetheless, we believe that the present novel observations apprehend the understanding of the nandrolone-induced mitigation of fatty infiltration and atrophy of the released rotator cuff muscle and the loss of this response when administration starts after fatty atrophy has been established.

4.3. Overview of alterations across biological layers

The comparison of the changes in muscle composition and transcript expression 16 weeks after tendon release revealed that these did correspond for factors being associated with muscle fibers and the extracellular matrix. For instance the down-regulation of GOs associated with muscle contraction did coincide with the reduction in the area percentage of muscle fibers

Table 3
Gene ontologies demonstrating altered responsiveness to nandrolone when administered after tendon release.
Counts, and *p*-values of the changes with nandrolone administration in the respective group for gene ontologies which transcripts demonstrated significant opposite regulation with nandrolone administration respective to CONTROL during the response to tendon release (N22W) or during the response to repair (N6W).

		N22W (TR vs. PRE) vs. CONTROL (TR vs. PRE)				N6W (END vs. TR) vs. CONTROL (END vs. TR)			
Processes	counts entire GO	trend	p-value	counts affected	counts opposite trend	trend	p-value	counts affected	counts opposite trend
Fat									
cellular lipid metabolic process	1163	up	1.90E−02	126	0	down	7.20E−21	117	11
glycerolipid metabolic process	419	up	1.90E−02	51	0	down	5.70E−10	49	2
fatty acid metabolic process	412	up	2.40E−03	55	0	down	2.90E−08	52	5
phospholipid metabolic process	390	up	7.90E−03	50	0	down	1.70E−08	47	3
positive regulation of lipid metabolic process	200	up	2.20E−05	37	0	down	8.30E−05	36	4
glycerophospholipid metabolic process	316	up	1.20E−02	41	0	down	3.70E−07	39	2
regulation of lipid biosynthetic process	222	up	8.50E−06	41	0	down	6.90E−04	37	6
glycerolipid biosynthetic process positive regulation of lipid	268	up	1.10E−02	36	0	down	9.20E−06	34	2
biosynthetic process	119	up	5.50E−05	25	0	down	2.60E−03	25	2
phospholipid biosynthetic process	280	up	2.10E−02	36	0	down	9.20E−06	34	2
glycerophospholipid biosynthetic process	240	up	1.80E−02	32	0	down	1.20E−04	30	2
lipid modification	185	up	6.00E−04	31	0	down	8.40E−03	28	4
Skeletal muscle									
muscle system process	376	up	4.90E−11	75	0	down	5.30E−11	68	7
regulation of muscle system process	293	up	6.40E−08	56	0	down	5.10E−09	52	4
muscle contraction	299	up	5.30E−08	57	0	down	2.50E−07	51	6
muscle structure development	703	up	6.50E−06	99	0	down	1.60E−07	82	20
regulation of muscle contraction	223	up	6.40E−07	44	0	down	7.70E−06	40	4
muscle cell differentiation	421	up	9.40E−05	62	0	down	4.20E−07	55	8
striated muscle contraction	125	up	5.60E−07	30	0	down	4.10E−04	28	2
muscle tissue development	454	up	1.20E−03	61	0	down	2.90E−04	51	12
striated muscle tissue development	431	up	9.20E−04	59	0	down	7.60E−04	49	12
muscle cell development	218	up	3.10E−04	36	0	down	3.20E−03	32	5
cardiac muscle tissue development	258	up	2.80E−04	41	0	down	6.10E−03	35	7
positive regulation of muscle cell differentiation	134	up	9.20E−04	24	0	down	8.90E−03	23	2
regulation of striated muscle contraction	124	up	1.70E−03	22	0	down	5.20E−03	21	1
Extracellular matrix									
cell adhesion	1331	up	1.20E−05	166	0	down	2.80E−13	137	34
biological adhesion	1344	up	9.00E−06	168	0	down	4.30E−13	138	35
single organism cell adhesion	684	up	2.00E−06	99	0	down	5.40E−09	85	18
single organismal cell–cell adhesion	650	up	6.50E−06	93	0	down	1.70E−09	81	15
homotypic cell–cell adhesion	417	up	6.80E−07	69	0	down	1.80E−08	63	9
response to extracellular stimulus	800	up	1.30E−03	98	0	down	1.20E−11	88	14
regulation of cell adhesion	861	up	1.50E−03	104	0	down	1.40E−10	90	17
leukocyte cell–cell adhesion	400	up	2.40E−04	58	0	down	1.90E−07	54	7
regulation of cell–cell adhesion	537	up	3.00E−03	68	0	down	3.40E−07	60	10
cellular response to extracellular stimulus	313	up	4.00E−04	47	0	down	2.80E−04	43	8
negative regulation of cell adhesion	309	up	3.10E−03	43	0	down	1.30E−03	38	7
vasculature									
angiogenesis	426	up	7.50E−03	54	0	down	6.70E−06	50	8
regulation of smooth muscle cell proliferation	176	up	5.50E−04	30	0	down	3.80E−03	27	3
protein turnover									
regulation of proteolysis	939	up	4.20E−03	109	0	down	3.60E−12	97	17
post-translational protein modification	296	up	4.00E−04	45	0	down	4.00E−07	42	3
positive regulation of proteolysis	435	up	2.40E−04	62	0	down	8.70E−07	56	9
regulation of proteolysis involved in cellular protein catabolic process	340	up	1.40E−03	48	0	down	1.40E−05	44	6
positive regulation of proteolysis involved in cellular protein catabolic process	187	up	7.20E−04	31	0	down	8.40E−03	28	

(compare Table S1 with Fig. 1G and Fig. 5B). Similarly the up-regulation of GOs associated with the extracellular matrix matched the increase in area percentage changes of the extracellular matrix compartment (compare Table S1 with Fig. 1I and Fig. 5B). In contrast, changes in the lipidome and lipid area percentage after tendon release (and repair) were not reflected by co-directional level alterations in transcripts being related to lipid metabolism (compare Fig. 1H and Fig. 5B). For instance tendon release and repair elevated the pool size of phospholipids, cholesterols and fatty acyls consistent with reported alterations of lipid classes in

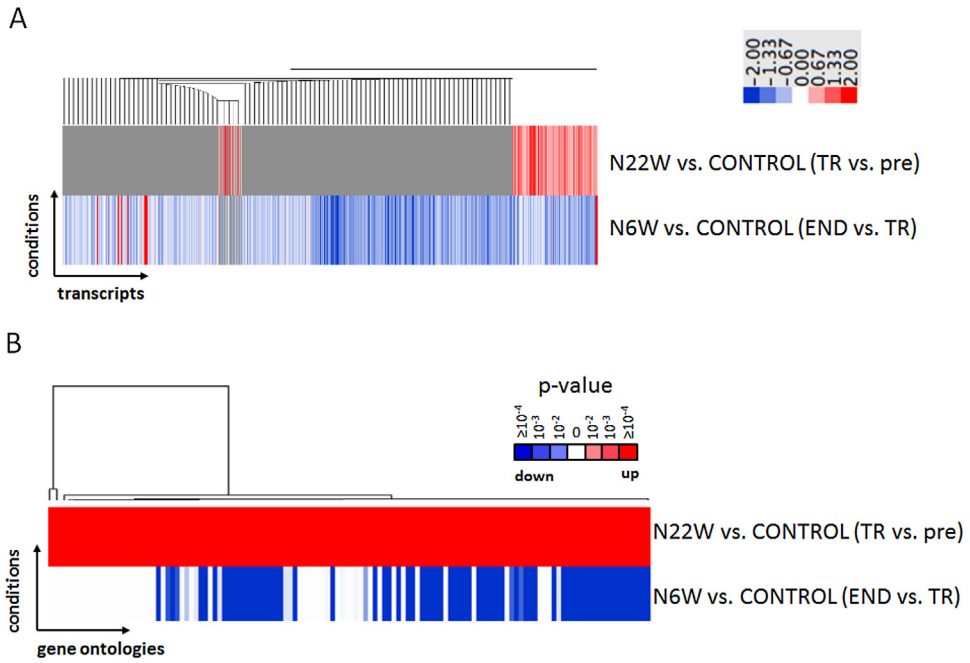

Fig. 6. Differences in the nandrolone-modulated transcriptome in function of administration. A) Heat map visualizing the regulation of the 10998 affected gene transcripts which altered their concentration after tendon release in N22W and/or repair in N6W (2% FDR based on SAM). B) Heat map visualizing the *p*-value weighed regulation of the 128 affected gene ontologies (p-value enrichment < 1% <FDR (Metacore) for altered transcripts (1% FDR based on SAM) which response differed 16 weeks after tendon release between nandrolone administration (N22W) vs. CONTROL or 6 weeks after repair (N6W) vs. CONROL. Changes of affected gene transcripts (A) and *p*-values for affected gene ontologies (B) are given in color-coding (red: up-regulated, blue: down-regulated). Non-significant changes are given in grey color.

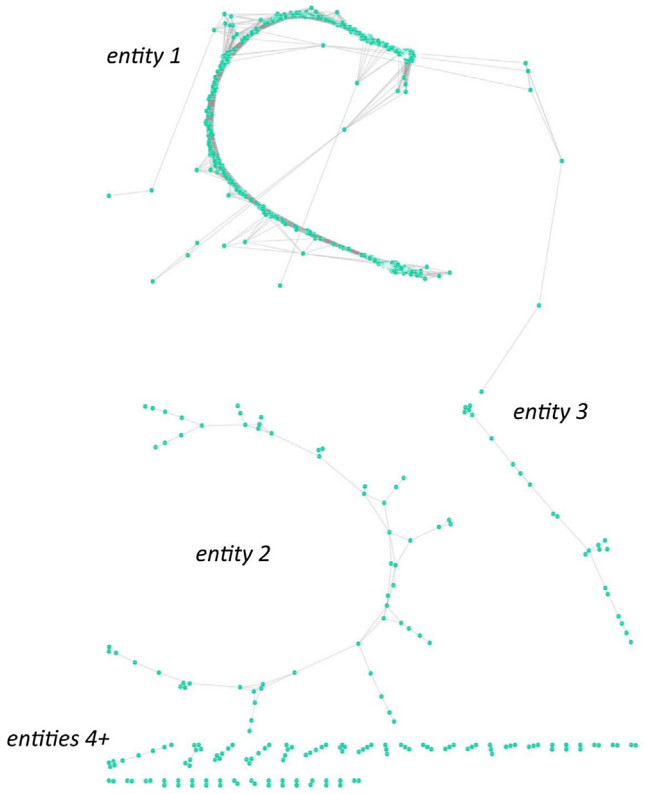

Fig. 7. Correlation network for alterations in lipid compounds and transcripts. Line graph displaying the correlations (nodes) between fold alterations in affected lipid compounds and transcripts (edges), which met the criteria of r > |0.99| for all studied samples in the three intervention groups. Nodes appear in turquoise and edges are displayed in grey. Identified entities are assigned. Entity 1 describes lipid compounds (654 nodes, 39674 edges), entities 2 (39 nodes, 77 edges) and 3 (14 nodes, 26 edges) describes only gene transcripts.

rabbit muscle after 1–2 weeks of tenotomy [24] and the lipid composition of adipocytes [42]. However, GOs associated with lipid biosynthesis were lowered 16 weeks after tendon release (at repair) and only few transcripts related to adipocyte differentiation were increased (Fig. 5B). Aside isoform 1 and 2 of the adipocyte differentiation marker PPARG, this included a 10-fold increase in negative (adiponectin; ADIPOQ, adipocyte enhancer binding protein 1; AEBP1) and positive regulators of adipogenesis (adipogenesis regulatory factor; ADIRF), the factor of fatty acid uptake and transport in adipocytes, fatty acid binding protein 4 (FABP4), and acyl-CoA synthetase medium-chain family member 1 (ACSM1). Other established markers of fat cell differentiation [43,44] that are increasingly expressed in rotator cuff muscle after tendon release, such as sterol regulatory element-binding protein 1 (SREBP1) and CCAAT/enhancer binding protein beta (C/EBPβ) [16,18], were not affected at the transcript level (data not shown). An up-regulation of lipid-associated gene transcripts, including PPARG, has been observed between 30 days and 16 weeks of tenotomy [18,41,45]. We corroborate this observation at the protein level by showing increased PPARG2 protein levels after 16 weeks of tendon release (Fig. 3A–C). Our findings support previous results in the rat, showing that fat accumulation with release of the rotator cuff tendon does not occur by activation of the canonical intramyocellular lipid storage and synthesis pathways [45] but does involve expressional up-regulation of specific pro-adipogenic factors.

4.4. Nandrolone and transcript regulation related to lipid biosynthesis

In contrast to the expectation from the lowered abundance of 243 lipid species (Table 2), nandrolone administration up-regulated transcript level for factors of fat cell differentiation and lipid biogenesis (Fig. 5B). For instance expression of transcripts being associated with central aspects of adipocyte differentiation (PPARG2; [46,47] and the maturation of lipid droplets (perilipin 1; PLIN1, vimentin, VIM) and FABP4 [48,49] remained unaffected by

nandrolone, i.e. it remained elevated at TR in the N22W group. We have shown previously that fat cell accumulation in infraspinatus muscle of sheep continues beyond 16 weeks of tendon release [3]. Collectively this implies that the prevention of fat accumulation in released infraspinatus muscle is not explained by a block of adipocyte differentiation, transcriptional down-regulated of lipid biosynthesis or lipid oxidation. Likely candidate explanations contributing to the nandrolone-mediated suppression of lipid biosynthesis, include the post-translational regulation of lipid biosynthesis in infraspinatus muscle or processes external to the studied muscle that control the delivery and import of substrates for lipid synthesis [7,39].

4.5. Nandrolone and androgen receptor expression

Our findings show that nandrolone administration with tendon release did up-regulate anabolic receptor protein and total RNA concentration in infraspinatus muscle of sheep (Fig. 3D/E, Fig. 4). This observation is consistent with previous findings on the effect of anabolic steroid on androgen receptor levels in mice, rat and humans [28,29,40,50]. Our findings show that nandrolone up-regulated transcripts of muscle-associated gene ontologies in line with a mitigated reduction of the area percentage in muscle fibers (Fig. 1G). This included prominent factors associated with contraction such as troponin C and I, myosin regulatory light chain and actinin, as well as myoglobin. We interpret our findings to indicate that the nandrolone-induced increase in androgen receptor levels are part of a feed forward mechanism that promotes the genomic effect of androgen signalling on protein metabolism in muscle fibers.

4.6. Tendon release affects the transcript response to nandrolone

Intriguingly, the absent effect of nandrolone on lipid accumulation when administration started after repair (N6W) was related to the inversion of the global transcript response compared to the N22W group when nandrolone was given for the duration of release (Fig. 6). This result emphasized the reduced responsiveness of degenerated rotator cuff muscle to stimulation by nandrolone [30]. As well it was found that androgen receptor levels were not modified compared to the CONTROL group in the N6W group (Fig. 3E). These observations suggest that the reduced responsiveness of degenerated rotator cuff muscle to nandrolone is related to a down-regulation of the genomic response to anabolic steroid. In this regard it is of interest that androgen receptor density in rat skeletal muscle is increased by contractile activity and decreased by steroid treatment [51]. The unaffected androgen receptor levels in the N6W group imply that this disparity of effect involves adjustments in the capacity for androgen receptor mediated signalling in skeletal muscle.

4.7. Relationship between the nandrolone-modulated muscle metabolome and transcriptome

A considerable portion of the lipid species, of which an increased pool size after tenotomy was prevented by nandrolone, are found in plasma membranes, i.e. glycerolipid, phospholipid, prenol lipid, sphingolipid, sterol lipid. Nandrolone has been shown before to acutely affect the plasma membrane of many cell types during its cell entry, i.e. by exerting electrophysiological actions [52] and influencing lipid peroxidation [53]. The present novel observations raise the possibility that the mitigating effect of nandrolone on lipid accumulation in tenotomised infraspinatus muscle also involves direct effects on plasma membrane integrity. Network analysis (Fig. 7) identifies an important number of linear relationships within the class of lipid compounds, and gene transcripts, including those being associated with the regulation of lipid metabolism, but not between them. The role of affected plasma membrane species in respect to the changes in transcript expression following the early administration of the synthetic lipid compound, nandrolone, remains to be tested.

4.8. Clinical relevance

Our results provide a number of clinically relevant conclusions. The nandrolone- and repair-regulated concentration of the androgen receptor levels and downstream effects on transcript expression in infraspinatus muscle provides a signpost when the treatment effects are to be expected. The findings thereby emphasize that the specific importance of muscle activity regulated androgen receptor expression lies in the modulation of the effects of nandrolone on muscle fat content [29,51]. As well we noted that elevated protein expression of PPARG2 after 16 weeks of tendon release resisted to nandrolone administration. This suggests that differentiation of adipocytes in released rotator cuff muscle may be irreversibly enhanced within the investigated time frame. As well we noted an important up-regulation of transcripts for adhesive processes, which are a prerequisite for adipocyte differentiation [44,54,55], with tendon release and this was further enhanced by nandrolone. This evidence for an enhanced presence of gene messages for pro-adipogenic processes bears the question for how long the released infraspinatus muscle is protected from lipid accumulation once nandrolone administration is discontinued. Finally, we identify at the level of statistical significance that nandrolone also reduces the loss in muscle fiber area percentage; supporting that nandrolone administration immediately after tendon release preserves muscle quality by avoiding the degeneration of contractile material.

5. Conclusion

Our molecular observations imply that the lost responsiveness of retracted rotator cuff muscle to anabolic steroid in lowering the pools sizes of lipid compounds is associated with defective, genomic actions of nandrolone, as reflected by a general down-regulation of transcript expression. Conversely, the prevention of lipid synthesis in sheep infraspinatus muscle with nandrolone administration starting immediately after tendon release is associated with up-regulated androgen receptor protein and RNA expression. Resistance of the adipocyte marker PPARG to regulation by nandrolone, indicates that prevention of fat accumulation in retracting rotator cuff muscle by nandrolone administration is not mediated by the suppression of adipocyte differentiation.

Conflict of interest

The authors have no conflict of interest.

Funding

The authors acknowledge a grant from RESORTHO and grant 310030_149786 from the Swiss National Science Foundation to MF.

Acknowledgments

The study was conducted with technical assistance of MSc Céline Ferrié. The proficient technical assistance of Drs. Anna Bratus and Lennart Opitz during transcriptome analysis is acknowledged.

Authors contributions

Conception and design of research: KW, CG, MF; Performed animal experiments: KW, DM, BR, MB; Performed muscle analysis: SR, CM, PV, EL, JH; Analyzed data: SR, PV, KW, CM, EL, MF; Interpreted results of experiments: MF, SR, EL; Funding: MF, CG; Prepared figures: SR, MF; Drafted manuscript: MF; Edited and revised manuscript: MF, SR, CG; Approved final version of manuscript; All authors. Abbreviations: SR, Severin Ruoss; CM, Christoph B Möhl; PV, Paola Valdivieso; MF, Martin Flück; EL, Endre Laczko; JH, Junmin Hu; KW, Karl Wieser; DM, Dominik C Meyer; CG, Christian Gerber; BR, Brigitte von Rechenberg; MB, Mario C Benn.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2016.08.005.

References

[1] J.S. Sher, J.W. Uribe, A. Posada, B.J. Murphy, M.B. Zlatkin, Abnormal findings on magnetic resonance images of asymptomatic shoulders, J. Bone Joint Surg. Am. 77 (1) (1995) 10–15.

[2] K. Yamaguchi, K. Ditsios, W.D. Middleton, C.F. Hildebolt, L.M. Galatz, S.A. Teefey, The demographic and morphological features of rotator cuff disease. A comparison of asymptomatic and symptomatic shoulders, J. Bone Joint Surg. Am. 88 (8) (2006) 1699–1704.

[3] C. Gerber, D.C. Meyer, A.G. Schneeberger, H. Hoppeler, B. von Rechenberg, Effect of tendon release and delayed repair on the structure of the muscles of the rotator cuff: an experimental study in sheep, J. Bone Joint Surg. Am. 86-A (9) (2004) 1973–1982.

[4] C. Gerber, D.C. Meyer, E. Frey, B. von Rechenberg, H. Hoppeler, R. Frigg, B. Jost, M.A. Zumstein, Neer Award 2007: Reversion of structural muscle changes caused by chronic rotator cuff tears using continuous musculotendinous traction. An experimental study in sheep, J. Shoulder Elbow Surg. 18 (2) (2009) 163–171.

[5] V.M. Wang, Important preliminary findings on the potential role for nandrolone decanoate in the treatment of chronic rotator cuff tears, J. Bone Joint Surg. Am. 93 (23) (2011) e1441–1442.

[6] V. Matyash, G. Liebisch, T.V. Kurzchalia, A. Shevchenko, D. Schwudke, Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics, J. Lipid Res. 49 (5) (2008) 1137–1146.

[7] M. Alen, P. Rahkila, Anabolic-androgenic steroid effects on endocrinology and lipid metabolism in athletes, Sports Med. 6 (6) (1988) 327–332.

[8] Z. Laron, A. Kowadlo-Silbergeld, Further evidence for a fat mobilizing effect of androgens, Acta Edocrinol. 45 (1964) 427–436.

[9] S. Bhasin, R. Jasuja, Selective androgen receptor modulators as function promoting therapies, Curr. Opin. Clin. Nutr. Metab. Care 12 (3) (2009) 232–240.

[10] C. Gerber, D.C. Meyer, K.M. Nuss, M. Farshad, Anabolic steroids reduce muscle damage caused by rotator cuff tendon release in an experimental study in rabbits, J. Bone Joint Surg. Am. 93 (23) (2011) 2189–2195.

[11] C. Gerber, D.C. Meyer, M. Fluck, M.C. Benn, B. von Rechenberg, K. Wieser, Anabolic steroids reduce muscle degeneration associated with rotator cuff tendon release in sheep, Am. J. Sports Med. 43 (10) (2015) 2393–2400.

[12] L. Burch, J.R. Scaife, H. Galbraith, Effect of anabolic steroids on lipogenic and lipolytic enzymes in sheep tissues, Horm. Metab. Res. 14 (1) (1982) 52–53.

[13] K. Blouin, M. Nadeau, M. Perreault, A. Veilleux, R. Drolet, P. Marceau, J. Mailloux, V. Luu-The, A. Tchernof, Effects of androgens on adipocyte differentiation and adipose tissue explant metabolism in men and women, Clin. Endocrinol. (Oxf.) 72 (2) (2010) 176–188.

[14] W. Qin, J. Pan, W.A. Bauman, C.P. Cardozo, Differential alterations in gene expression profiles contribute to time-dependent effects of nandrolone to prevent denervation atrophy, BMC Genomics 11 (2010) 596.

[15] V. Dubois, M. Laurent, S. Boonen, D. Vanderschueren, F. Claessens, Androgens and skeletal muscle: cellular and molecular action mechanisms underlying the anabolic actions, Cell. Mol. Life Sci. 69 (10) (2012) 1651–1667.

[16] S.K. Joshi, X. Liu, S.P. Samagh, D.H. Lovett, S.C. Bodine, H.T. Kim, B.T. Feeley, mTOR regulates fatty infiltration through SREBP-1 and PPARgamma after a combined massive rotator cuff tear and suprascapular nerve injury in rats, J. Orthop. Res. 31 (5) (2013) 724–730.

[17] A. Wyce, Y. Bai, S. Nagpal, C.C. Thompson, Research Resource: the androgen receptor modulates expression of genes with critical roles in muscle development and function, Mol. Endocrinol. 24 (8) (2010) 1665–1674.

[18] E. Frey, F. Regenfelder, P. Sussmann, M. Zumstein, C. Gerber, W. Born, B. Fuchs, Adipogenic and myogenic gene expression in rotator cuff muscle of the sheep after tendon tear, J. Orthop. Res. 27 (4) (2009) 504–509.

[19] J. Farup, L. Madaro, P.L. Puri, U.R. Mikkelsen, Interactions between muscle stem cells, mesenchymal-derived cells and immune cells in muscle homeostasis, regeneration and disease, Cell Death Dis. 6 (2015) e1830.

[20] C.C. Agley, A.M. Rowlerson, C.P. Velloso, N.R. Lazarus, S.D. Harridge, Human skeletal muscle fibroblasts, but not myogenic cells, readily undergo adipogenic differentiation, J. Cell Sci. 126 (Pt. 24) (2013) 5610–5625.

[21] T. Hosoyama, N. Ishiguro, K. Yamanouchi, M. Nishihara, Degenerative muscle fiber accelerates adipogenesis of intramuscular cells via RhoA signaling pathway, Differentiation 77 (4) (2009) 350–359.

[22] A. Sorisky, A.S. Molgat, A. Gagnon, Macrophage-induced adipose tissue dysfunction and the preadipocyte: should I stay (and differentiate) or should I go? Adv. Nutr. 4 (1) (2013) 67–75.

[23] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, Molecular Biology of the Cell, 4th edition, Garland Science, New York, 2002.

[24] L. Heiner, J. Domonkos, M. Vargha Jr., Effect of tenotomy on the lipid composition of muscles with different biological functions, Acta Physiol. Acad. Sci. Hung. 45 (3–4) (1974) 163–171.

[25] S.A. Petersen, T.P. Murphy, The timing of rotator cuff repair for the restoration of function, J. Shoulder Elbow Surg. 20 (1) (2011) 62–68.

[26] H.E. Lahteenmaki, P. Virolainen, A. Hiltunen, J. Heikkila, O.I. Nelimarkka, Results of early operative treatment of rotator cuff tears with acute symptoms, J. Shoulder Elbow Surg. 15 (2) (2006) 148–153.

[27] A.G. Fragkaki, Y.S. Angelis, M. Koupparis, A. Tsantili-Kakoulidou, G. Kokotos, C. Georgakopoulos, Structural characteristics of anabolic androgenic steroids contributing to binding to the androgen receptor and to their anabolic and androgenic activities. Applied modifications in the steroidal structure, Steroids 74 (2) (2009) 172–197.

[28] J.A. Carson, W.J. Lee, J. McClung, G.A. Hand, Steroid receptor concentration in aged rat hindlimb muscle: effect of anabolic steroid administration, J. Appl. Physiol. 93 (1) (1985) 42–250 (2002).

[29] W.J. Lee, J. McClung, G.A. Hand, J.A. Carson, Overload-induced androgen receptor expression in the aged rat hindlimb receiving nandrolone decanoate, J. Appl. Physiol. 94 (3) (1985) 153–1161 (2003).

[30] C. Gerber, D.C. Meyer, B. Von Rechenberg, H. Hoppeler, R. Frigg, M. Farshad, Rotator cuff muscles lose responsiveness to anabolic steroids after tendon tear and musculotendinous retraction: an experimental study in sheep, Am. J. Sports Med. 40 (11) (2012) 2454–2461.

[31] M. Flueck, N. Eyeang-Bekale, A. Heraud, A. Girard, M. Gimpl, O.R. Seynnes, J. Rittweger, J. Niebauer, E. Mueller, M. Narici, Load-sensitive adhesion factor expression in the elderly with skiing: relation to fiber type and muscle strength, Scand. J. Med. Sci. Sports 21 (Suppl. 1) (2011) 29–38.

[32] J.M. Buescher, S. Moco, U. Sauer, N. Zamboni, Ultrahigh performance liquid chromatography–tandem mass spectrometry method for fast and robust quantification of anionic and aromatic metabolites, Anal. Chem. 82 (11) (2010) 4403–4412.

[33] S. Schmutz, C. Dapp, M. Wittwer, A.C. Durieux, M. Mueller, F. Weinstein, M. Vogt, H. Hoppeler, M. Fluck, A hypoxia complement differentiates the muscle response to endurance exercise, Exp. Physiol. 95 (6) (2010) 723–735.

[34] A. Dobin, C.A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, T.R. Gingeras, STAR: ultrafast universal RNA-seq aligner, Bioinformatics 29 (1) (2013) 15–21.

[35] S. Klossner, R. Li, S. Ruoss, A.C. Durieux, M. Fluck, Quantitative changes in focal adhesion kinase and its inhibitor, FRNK, drive load-dependent expression of costamere components, Am. J. Physiol. Regul. Integr. Comp. Physiol. 305 (6) (2013) R647–657.

[36] V.G. Tusher, R. Tibshirani, G. Chu, Significance analysis of microarrays applied to the ionizing radiation response, Proc. Natl. Acad. Sci. U. S. A. 98 (9) (2001) 5116–5121.

[37] J. Lessard, M. Pelletier, L. Biertho, S. Biron, S. Marceau, F.S. Hould, S. Lebel, F. Moustarah, O. Lescelleur, P. Marceau, A. Tchernof, Characterization of dedifferentiating human mature adipocytes from the visceral and subcutaneous fat compartments: fibroblast-activation protein alpha and dipeptidyl peptidase 4 as major components of matrix remodeling, PLoS One 10 (3) (2015) e0122065.

[38] D. Goutallier, J.M. Postel, P. Gleyze, P. Leguilloux, S. Van Driessche, Influence of cuff muscle fatty degeneration on anatomic and functional outcomes after simple suture of full-thickness tears, J. Shoulder Elbow Surg. 12 (6) (2003) 550–554.

[39] C. Mora, M.L. Macia, J. Garcia, J.F. Navarro, Effect of nandrolone decanoate on the lipid profile of male peritoneal dialysis patients, Perit. Dial. Int. 21 (6) (2001) 611–614.

[40] M. Galbiati, E. Onesto, A. Zito, V. Crippa, P. Rusmini, R. Mariotti, M. Bentivoglio, C. Bendotti, A. Poletti, The anabolic/androgenic steroid nandrolone exacerbates gene expression modifications induced by mutant SOD1 in muscles of mice models of amyotrophic lateral sclerosis, Pharmacol. Res. 65 (2) (2012) 221–230.

[41] H.M. Kim, L.M. Galatz, C. Lim, N. Havlioglu, S. Thomopoulos, The effect of tear size and nerve injury on rotator cuff muscle fatty degeneration in a rodent animal model, J. Shoulder Elbow Surg. 21 (7) (2012) 847–858.

[42] K. Tauchi-Sato, S. Ozeki, T. Houjou, R. Taguchi, T. Fujimoto, The surface of lipid droplets is a phospholipid monolayer with a unique fatty acid composition, J. Biol. Chem. 277 (46) (2002) 44507–44512.

[43] L. Fajas, D. Auboeuf, E. Raspe, K. Schoonjans, A.M. Lefebvre, R. Saladin, J. Najib, M. Laville, J.C. Fruchart, S. Deeb, A. Vidal-Puig, J. Flier, M.R. Briggs, B. Staels, H. Vidal, J. Auwerx, The organization, promoter analysis, and expression of the human PPARgamma gene, J. Biol. Chem. 272 (30) (1997) 18779–18789.

[44] A.G. Cristancho, M.A. Lazar, Forming functional fat: a growing understanding of adipocyte differentiation, Nat. Rev. Mol. Cell Biol. 12 (11) (2011) 722–734.

[45] J.P. Gumucio, M.A. Korn, A.L. Saripalli, M.D. Flood, A.C. Phan, S.M. Roche, E.B. Lynch, D.R. Claflin, A. Bedi, C.L. Mendias, Aging-associated exacerbation in fatty degeneration and infiltration after rotator cuff tear, J. Shoulder Elbow Surg. 23 (1) (2014) 99–108.
[46] A.V. Hertzel, L.A. Smith, A.H. Berg, G.W. Cline, G.I. Shulman, P.E. Scherer, D.A. Bernlohr, Lipid metabolism and adipokine levels in fatty acid-binding protein null and transgenic mice, Am. J. Physiol. Endocrinol. Metab. 290 (5) (2006) E814–E823.
[47] L. Wang, L. Li, J. Jiang, Y. Wang, T. Zhong, Y. Chen, H. Zhang, Molecular characterization and different expression patterns of the FABP gene family during goat skeletal muscle development, Mol. Biol. Rep. 42 (1) (2015) 201–207.
[48] H. Heid, S. Rickelt, R. Zimbelmann, S. Winter, H. Schumacher, Y. Dorflinger, C. Kuhn, W.W. Franke, On the formation of lipid droplets in human adipocytes: the organization of the perilipin-vimentin cortex, PLoS One 9 (2) (2014) e90386.
[49] K. Hsieh, Y.K. Lee, C. Londos, B.M. Raaka, K.T. Dalen, A.R. Kimmel, Perilipin family members preferentially sequester to either triacylglycerol-specific or cholesteryl-ester-specific intracellular lipid storage droplets, J. Cell Sci. 125 (Pt. 17) (2012) 4067–4076.
[50] A.A. Ferrando, M. Sheffield-Moore, C.W. Yeckel, C. Gilkison, J. Jiang, A. Achacosa, S.A. Lieberman, K. Tipton, R.R. Wolfe, R.J. Urban, Testosterone administration to older men improves muscle function: molecular and physiological mechanisms, Am. J. Physiol. Endocrinol. Metab. 282 (3) (2002) E601–607.
[51] V.A. Bricout, P.S. Germain, B.D. Serrurier, C.Y. Guezennec, Changes in testosterone muscle receptors: effects of an androgen treatment on physically trained rats, Cell Mol. Biol. (Noisy-le-grand) 40 (3) (1994) 291–294.
[52] M. Estrada, A. Espinosa, M. Muller, E. Jaimovich, Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells, Endocrinology 144 (8) (2003) 3586–3597.
[53] T. Nikolic, V. Zivkovic, M. Jevdjevic, M. Djuric, I. Srejovic, D. Djuric, N. Jeremic, S. Bolevich, V. Jakovljevic, The effects of chronic administration of nandrolone decanoate on redox status in exercised rats, Mol. Cell. Biochem. 411 (1–2) (2016) 95–105.
[54] I. Titushkin, S. Sun, A. Paul, M. Cho, Control of adipogenesis by ezrin, radixin and moesin-dependent biomechanics remodeling, J. Biomech. 46 (3) (2013) 521–526.
[55] J.J. Li, D. Xie, Cleavage of focal adhesion kinase (FAK) is essential in adipocyte differentiation, Biochem. Biophys. Res. Commun. 357 (3) (2007) 648–654.