Running Head: Human Muscle Proteome Responses to a High Fat Diet and Exercise

# Dynamic Proteome Profiling of Individual Proteins in Human Skeletal Muscle Following A High Fat Diet & Resistance Exercise

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**Abbreviations:**

4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1; ACTS, Actin Alpha 1; ALBU, Albumin; AMPK, 5' AMP-activated protein kinase; ANKR2, ankyrin repeat domain protein; CAH3, carbonic anhydrase 3; CRYAB, alpha B-crystallin; $D_2O$, Deuterium oxide; DPP, Dynamic Proteome Profiling; FAT/CD36, fatty acid translocase; FFA, Free fatty acids; FFM, Fat free mass; FLNC, Filamin-C; GPR56, G protein-coupled receptor 56; HFLC, High Fat Low Carbohydrate; HSPB1, heat shock protein; IL-6, Interleukin-6; IQR, inter-quartile range; MAFbx, Muscle Atrophy F-box; MyHC, myosin heavy chain; MPE, molar per-cent enrichment; MuRF1, Muscle-specific RING Finger protein-1; mTOR, mechanistic target of rapamycin; OGTT, Oral Glucose Tolerance Test; ODPB, Pyruvate dehydrogenase E1 component subunit beta; PAI-1, Plasminogen activator inhibitor type 1; PDK4, Pyruvate dehydrogenase lipoamide kinase isozyme 4; PEBP1, Phosphatidylethanolamine-binding protein 1; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha PRDX6, Peroxiredoxin-6; PROF1, Profilin-1; REX, Resistance Exercise; RKIP, RAF kinase inhibitor protein; TNNC2, Troponin C; TNF-α, Tissue Necrosis Factor-α; TRFE, Transferrin

**Abstract**

It is generally accepted that muscle adaptation to resistance exercise training is underpinned by contraction-induced increased rates of protein synthesis and dietary protein availability. Utilising Dynamic Proteome Profiling (DPP), we investigated the contributions of both synthesis and breakdown to changes in abundance on a protein-by-protein basis in human skeletal muscle. Age-matched, overweight males consumed nine days of a high-fat, low-carbohydrate (HFLC) diet during which time they either undertook three sessions of resistance exercise (REX) or performed no exercise. Precursor enrichment and the rate of incorporation of deuterium oxide ($D_2O$) into newly synthesised muscle proteins were determined by mass spectrometry. Ninety proteins were included in the DPP with 28 proteins exhibiting significant responses to REX. The most common pattern of response was an increase in turnover, followed by an increase in abundance with no detectable increase in protein synthesis. We provide novel evidence demonstrating that the contribution of synthesis and breakdown to changes in protein abundance induced by REX differ on a protein-by-protein basis. We also highlight the importance of the degradation of individual muscle proteins following exercise in human skeletal muscle.

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Skeletal muscle displays remarkable plasticity with the capacity to alter its phenotype in response to contractile activity and nutrient availability (1, 2). Resistance exercise (REX) increases muscle size and contractile strength and is an important intervention to prevent the muscle loss associated with disuse, ageing or disease-related cachexia (2). Exercise-induced gains in muscle mass are commonly attributed to a greater protein synthetic response. Indeed, a single bout of REX increases muscle protein fractional synthetic rate ~20% above basal levels (3-5), a response further augmented with amino acid ingestion (6-8).

In humans the synthesis and degradation of muscle proteins *in vivo* has been extensively investigated via metabolic labelling experiments using sterile intra-venous infusions of stable isotopes such as [$^2H_5$]-phenylalanine (5, 9). However, the acute elevations in muscle protein synthesis estimated by these techniques do not always correlate with muscle hypertrophy responses induced by chronic REX (10, 11). Additionally, the acute nature of amino acid tracer methods (i.e. time course of < 12 hours) means changes in protein turnover during fasted (sleeping) periods and over several days are not captured. Metabolic labelling with deuterium oxide ($D_2O$) offers an elegant solution to investigate the chronic effects of exercise-nutrient interactions on rates of muscle protein synthesis and degradation because it can be administered via drinking water to free-living humans over prolonged periods (weeks to months) allowing measurement under 'real world' conditions of habitual diet and activity patterns (12). Using these techniques, Brook and colleagues recently reported an 18% increase in the synthesis rate of mixed myofibrillar proteins during the first 3 wk of a REX intervention that accounted for most of the observed increase in muscle mass (13). However, that study did not measure individual protein responses to exercise, so the relationship between an average increase in synthesis across mixed myofibrillar proteins and selective changes in myofibrillar protein abundance was not clear. A greater understanding of protein specific responses, including rates of synthesis, degradation and abundance, is important given that suppression of the rate of renewal of some proteins can result in aggregation, gain-of-function toxicity and a loss of proteome quality (proteostasis), a hallmark of ageing (14).

In a comprehensive study of muscle protein responses to exercise, we combine state-of-the-art measurements of protein synthesis rates with well-established label-free profiling methods (15-17) that are considered the "gold-standard" for measuring relative changes in protein abundance in humans. For the first time, we report the anabolic effects of REX on the synthesis, breakdown and abundance of individual muscle proteins in human skeletal muscle.

Specifically, we investigated whether a short-term high-fat, low carbohydrate (HFLC) diet impairs rates of muscle protein synthesis, degradation and abundance, as such diets are often promoted for rapid weight loss. However, a common shortcoming of HFLC diets is that they fail to confer protection against the debilitating loss of skeletal muscle mass (18) and therefore provide a suitable model to measure synthesis and degradation rates of individual muscle proteins.

## Materials and Methods

*Subjects* Sixteen sedentary, untrained, otherwise healthy male subjects were recruited for this study (Table 1). Participants were provided with oral and written information about the purpose, nature and potential risks involved with the study, and written informed consent was obtained prior to participation. The study was approved by the Australian Catholic University Human Research Ethics Committee and conformed with the policy statement regarding the use of human subjects in the latest revision of the Declaration of Helsinki. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN 369316). The study employed a between-subjects design where subjects were pair-matched for fat free mass and strength and allocated to either an exercise ($n$ = 8, REX) or non-exercise ($n$ = 8, HFLC) group for the experimental trial. Previous studies measuring individual muscle proteome responses in humans have been performed in only 3-6 participants (19, 20). Moreover, we did not allow any missing values in our proteomic analyses (proteins that could not be measured in all 16 participants at all time points were excluded).

*Preliminary Testing and Control Diet*: Preliminary Testing $VO_{2peak}$ and Maximum Strength were determined two-weeks prior to the commencement of experimental trials (described subsequently). $VO_{2peak}$ was determined during an incremental test to volitional fatigue on a Lode cycle ergometer (Groningen, The Netherlands) (21). Quadriceps strength was determined during a series of single repetitions on a pin-loaded leg extension (Cybex, Massachusetts, USA) and leg press (Synergy Leg Press, Queensland, Australia) machine until the maximum load lifted was established (1 RM) (6). Body composition (fat mass and fat free mass (FFM)) was measured using whole-body dual-energy x-ray absorptiometry (DXA) scans (GE Lunar iDXA Pro, enCORE software Version 16) under standardized conditions. $VO_{2peak}$, strength and body composition were determined two weeks prior to experimental trials during which time subjects maintained their habitual diet and physical activity patterns. Following preliminary testing, participants were instructed to refrain from exercise training,

vigorous physical activity, alcohol and caffeine consumption for the 72 h before commencing the 9-day high fat diet and exercise experimental period (described subsequently). During this time, participants were also provided with standardized prepacked meals for breakfast, lunch, dinner and snacks that provided an energy availability of 45 kcal·kg fat-free mass (FFM) $^{-1}$·day$^{-1}$ consisting of 6.1 g carbohydrate/kg FFM (55% caloric intake), 1.7 g protein/ kg FFM (15%) and 1.5 g fat/ kg FFM (30%) (Figure 1).

*High Fat Low Carbohydrate (HFLC) Diet*. On the next morning following the diet and exercise control period, participants reported to the laboratory after a ~10-h overnight fast. After resting in the supine position for ~15 min, a catheter was inserted into the antecubital vein of one arm and a baseline blood sample (~5 mL) was taken. Under local anaesthesia (2–3 mL of 1% Xylocaine) a resting biopsy (Day 1) was obtained from the *vastus lateralis* using a 5-mm Bergstrom needle modified with suction. An oral glucose tolerance test (OGTT) was then conducted using a 75 g 300 mL glucose solution with blood samples obtained every 30 min up to 2 h. Participants were then provided their HFLC diet to be consumed as their only caloric intake for the next 9 days consisting of 0.8g carbohydrate kg/ FFM (8% total caloric intake), 1.7 g protein/ kg FFM (15%) and 3.9 g fat/ kg FFM (77%) (Figure 1). This diet followed guidelines previously reported (22). Total energy intake was based on an energy availability of 45 kcal·kg FFM$^{-1}$·day$^{-1}$ to ensure participants were in energy balance, and meal plans were created using Foodworks 7.0 ® Xyris Software (Melbourne, Australia). Menu construction and the preparation of meals and snacks were undertaken by food service dietitians and sports dietitians (Dineamic, Camberwell, Victoria). Meal plans were individually developed for each participant to integrate individual food preferences and BM. All meals and snacks were supplied to subjects. Participants were required to keep a food checklist to note their compliance to the dietary instructions and their intake of any additional food or drinks. Every 2 days, participants met with a dietician to receive new food parcels and check their adherence to the previous days' diet. Additional muscle biopsies were obtained on Day 3 and 6 of the HFLC diet. Each muscle biopsy was taken under fasted conditions from a separate site 2-3 cm distal from the same leg and moving in a proximal direction with successive biopsies with all samples stored at -80°C until subsequent analysis. Blood samples were obtained every morning in EDTA tubes via venepuncture from the antecubital vein on alternate arms. All blood samples were immediately centrifuged at 1,000 g at 4°C for 15 min, with aliquots of plasma frozen in liquid $N_2$ and stored at −80°C. The

morning following the HFLC diet, an additional muscle biopsy was obtained and participants then underwent another OGTT as previously described.

*Deuterium Labelling Protocol* Deuterium labelling of newly synthesized proteins was achieved by oral consumption of $D_2O$ (Sigma Aldrich, Castle Hill, Australia) based on previous work (19). To achieve an appropriate target enrichment of 1–2%, participant's consumed 50 ml of 99.8 atom % $D_2O$ four times a day for a total of 200 mL per day for all days of the HFLC intervention commencing after the first muscle biopsy on Day 1. All 50 mL doses were provided in individually sealed bottles purchased from Sigma Aldrich and participants were instructed to consume each dose at least 3-4 h apart.

*Resistance Exercise* Participants in the REX group performed a resistance exercise session on Day 1, 4 and 7 of the HFLC diet. The resistance exercise session consisted of 4 X 8-10 repetitions of leg press at 80% 1-RM, 4 X 8-10 repetitions of leg extension at 80% 1-RM, and 4 sets of dumbbell squats. Each set was separated by a 3-min recovery period during which time participants rested.

*Gas chromatography-mass spectrometry* Body water enrichment of $D_2O$ was measured in plasma samples against external standards constructed by adding $D_2O$ to phosphate buffered saline over the range from 0.0 to 3.0 % in 0.5 % increments. The $D_2O$ enrichment of aqueous solutions was determined after exchange with acetone (23). Samples were centrifuged at 12,000 g, 4 ºC for 10 min and 20 µL of plasma supernatant or standard was reacted overnight at room temperature with 2 µL of 10 N NaOH and 4 µL of 5 % (v/v) acetone in acetonitrile. Acetone was then extracted in to 500 µL chloroform and water was captured in 0.5 g $Na_2SO_4$ prior to transferring a 200 µL aliquot of chloroform to an auto-sampler vial. Samples and standards were analysed in triplicate using an Agilent 5973N mass selective detector coupled to an Agilent 6890 gas chromatography system. A CD624-GC column (30 m x 0.25 mm x 1.40 µ m) was used in all analyses. Samples (1 µL) were injected using an Agilent 7683 auto sampler. The temperature program began at 50 °C and increased by 30 °C/min to 150 °C, and was held for 1 min. The split ratio was 50:1 with a helium flow of 1.5 mL/min. Acetone eluted at approximately 3.0 min. The mass spectrometer was operated in the electron impact mode (70 eV) and selective ion monitoring of m/z 58 and 59 was performed using a dwell time of 10 ms/ion.

*Muscle processing* Muscle samples (~60 mg) were pulverised in liquid nitrogen then homogenised on ice in 10 volumes of 1% Triton X-100, 50 mM Tris pH 7.4 containing Complete™ protease inhibitor (Roche Diagnostics, Lewes, UK) using a PolyTron homogeniser. Samples were incubated on ice for 15 min then centrifuged at 1,000 g, 4°C for 5 min. Supernatants containing soluble/ sarcoplasmic proteins were decanted and stored on ice while the myofibrillar pellet was resuspended in 0.5 ml of homogenisation buffer and then centrifuged at 1,000 g, 4°C for 5 min. The washed myofibrillar pellet was then solubilised in 0.5 ml of 7 M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris pH 8.5 and cleared by centrifugation at 12,000 g, 4°C for 45 min. The protein concentrations of both the myofibrillar fraction and sarcoplasmic fraction were measured using the Bradford assay (Sigma, Poole, Dorset, UK). Aliquots containing 100 µg protein were precipitated in 5 volumes of acetone for 1 hour at −20°C. Pellets were resuspended in 0.1% (w/v) Rapigest SF (Waters; Milford, MA, USA) in 50 mM ammonium bicarbonate and incubated at 80°C for 15 min. Samples were washed with 0.1% (w/v) Rapigest SF (Waters; Milford, MA, USA) in 50 mM ammonium bicarbonate using spin columns with 5 kDa molecular weight filters and adjusted to a final volume of 100 µL. DTT was added (final concentration 1 mM) and incubated at 60°C for 30 min followed by incubation (30 min) while being protected from light in the presence of 5 mM iodoacetamide at 4°C. Sequencing grade trypsin (Promega; Madison, WI, USA) was added at a protein ratio of 1:50 and digestion allowed to proceed at 37°C overnight. Digestion was terminated by the addition of 2 µL concentrated TFA and peptide solutions were cleared by centrifugation at 15,000 g for 15 min.

*Liquid chromatography-mass spectrometry* Label-free liquid chromatography-mass spectrometry (LC-MS) analysis was performed using nanoscale reversed-phase ultra-performance liquid chromatography (nanoACQUITY, Waters, Milford, MA) and online electrospray ionisation (ESI) quadrupole - time of flight mass spectrometry (Q-TOF Premier, Waters, Manchester, UK). Samples (400 ng tryptic peptides) were loaded in aqueous 0.1% (v/v) formic acid via a Symmetry C18 5 µm, 2 cm x 180 µm trap column (Waters, Milford, MA). Separation was conducted at 35 ºC through a BEH C18 1.7 µm, 25 cm x 75 µm analytical reverse phase column (Waters, Milford, MA). Peptides were eluted using a gradient rising to 37 % acetonitrile 0.1% (v/v) formic acid over 90 min at a flow rate of 300 nL/min. For all measurements, the mass spectrometer was operated in a data-dependent positive ESI mode at a resolution of >10,000 FWHM. Prior to analysis, the time of flight analyser was calibrated using fragment ions of [Glu-1]-fibrinopeptide B from m/z 50 to 1990.

Mass spectra for LC-MS profiling were recorded between 350 m/z and 1600 m/z using MS survey scans of 0.45 s duration with an inter-scan delay of 0.05 s. In addition, equivalent data-dependent tandem mass spectrometry (MS/MS) spectra were collected from each D0 (control) sample. MS/MS spectra of collision-induced dissociation fragment ions were recorded for the 5 most abundant precursor's ions of charge 2+ or 3+ detected in the survey scan. Precursor fragmentation was achieved by collision induced dissociation (CID) at an elevated (20-40 eV) collision energy over a duration of 0.15 s per parent ion with an inter-scan delay of 0.05 s over 50-2000 m/z. Acquisition was switched from MS to MS/MS mode when the base peak intensity (BPI) exceeded a threshold of 750 counts per second, and returned to the MS mode when the TIC in the MS/MS channel exceeded 50000 counts/s or when 1.0 s (5 scans) were acquired. To avoid repeated selection of peptides for MS/MS the program used a 30 s dynamic exclusion window.

*Progenesis protein profiling* Progenesis QI for proteomics (QI·P; Nonlinear Dynamics, Newcastle, UK) was used to perform label-free quantitation consistent with our previous work (15-17). Prominent ion features (>600 per chromatogram) were used as vectors to warp each dataset to a common reference chromatogram. An analysis window of 15 min - 105 min and 350 m/z - 1500 m/z was selected, which encompassed a total of 7,018 features with charge states of +2 or +3. Log transformed MS data were normalised by inter-sample abundance ratio and differences in relative protein abundance were investigated using non-conflicting peptides only. MS/MS spectra (48, 601 queries) were exported in Mascot generic format and searched against the Swiss-Prot database (2016.7) restricted to 'Homo Sapiens' (20,272 sequences) using a locally implemented Mascot (www.matrixscience.com) server (version 2.2.03). The enzyme specificity was trypsin allowing 1 missed cleavage, carbamidomethyl modification of cysteine (fixed), deamination of asparagine and glutamine (variable), oxidation of methionine (variable) and an m/z error of ± 0.3 Da. The Mascot output (xml format), restricted to non-homologous protein identifications was recombined with MS profile data and peptides modified by deamination or oxidation were removed prior to quantitative analysis.

*Measurement of protein synthesis rates* Protein synthesis rates were calculated using mass isotopomer distribution analysis consistent with previous work (19). Mass isotopomer abundance data were extracted from MS only spectra using Progenesis Quantitative Informatics (QI; Nonlinear Dynamics, Newcastle, UK). Peak picking was performed on ion

features with +1, +2 or +3 charge states within an analysis window of 15 min - 105 min and 350 m/z - 1500 m/z. The abundance of M0-M3 mass isotopomers were collected over the entire chromatographic peak for each non-conflicting peptide that was used for label-free quantitation in the aforementioned Progenesis QI·P analysis. Mass isotopomer information was exported from Progenesis QI and processed in R version 3.3.1 (24) according to published methods (19). Briefly, the incorporation of deuterium in to newly synthesised protein causes a decrease in the abundance (Figure 1) of the monoisotopic (M0) peak relative to the abundances of the M1, M2 and M3 isotopomers that contain 1, 2 or 3 'heavy' isotopes (e.g. 13C, $D_2O$, 15N, etc.). Over the duration of the experiment, changes in mass isotopomer distribution follow a non-linear bi-exponential pattern due to the rise-to-plateau kinetics in precursor enrichment (body water $D_2O$ enrichment measured in plasma samples by GC-MS) and the rise-to-plateau kinetics of $D_2O$-labelled amino acids in to newly synthesised protein. Therefore, a machine learning approach was taken employing the Nelder-Mead method to optimise for the rate of change in the relative abundance of the monoisotopic (M0) peak. The rate of change in mass isotopomer distribution is also a function of the number (n) of exchangeable H sites and this was accounted for by referencing each peptide sequence against standard tables reporting the relative enrichment of amino acids by tritium in mice (25) or deuterium in humans (26) to give the synthesis rate ($k$) for each peptide. Supplementary tables S1 and S2 report the raw mass spectrometry data for each peptide analysed in the myofibrillar and sarcoplasmic fractions, respectively. Peptide synthesis rates across each protein were averaged to give a synthesis rate for that protein in each individual participant.

*Estimation of protein breakdown rates*. The rate of change in the abundance of a protein is dependent on the difference between its rate of synthesis $k_s$ and rate of breakdown $k_d$. We assumed each of these was constant and used first-order kinetics following the standard formula:

$$A = A_0 e^{(k_s - k_d)(t - t_0)}$$

where $A$ is the abundance at time $t$ and $A_0$ is the abundance at time $t_0$. Using protein abundance data at times $t$ and $t_0$ the net rate of change in abundance can be calculated by rearranging the above to give:

$$k_s - k_d = \frac{1}{t - t_0} \ln \frac{A}{A_0}$$

Converting differences in abundance between D0 and D9 to rates of change in abundance enables the rate of breakdown of each protein to be calculated as the difference between its rate of synthesis and its rate of change in abundance. Supplementary table S3 summarises the mean (n=8 in each group) abundance, synthesis and degradation data for each protein investigated in the sarcoplasmic and myofibrillar fractions.

*Plasma Analysis* Plasma glucose concentration was measured via an enzymatic reference method with hexokinase (Melbourne Pathology, Melbourne, Australia). Plasma insulin, leptin, adiponectin, plasminogen activator inhibitor type 1 (PAI-1), tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) were measured on 96-well plates utilizing commercially available and customised Milliplex Human magnetic bead panels (Millipore, Massachusetts, USA) following the kit-specific protocols provided by Millipore. Analytes were quantified in duplicate using the Magpix system utilising xPONENT 4.2 software. Concentrations of all analytes were determined on the basis of the fit of a standard curve for mean fluorescence intensity versus pg/mL. Two quality controls with designated ranges were run with each assay to ensure validity of data generated. For the quantification of active ghrelin, whole blood samples (1 ml) were transferred to eppendorf tubes containing 1 mg 4-(2-aminoethyl)-benzene sulfonylfluoride (AEBSF; Sigma Aldrich, Castle Hill, Australia). Samples were then incubated at room temperature for 30 mins to clot and then centrifuged at 3,000g for 15 min at 4°C. Plasma samples were then acidified with 4 µL 1N Hydrochloric acid and then stored at -80°C. A sandwich ELISA (Millipore, Massachusetts, USA) incorporating spectrophotometric absorbance was used to measure active ghrelin levels. C-reactive protein levels were quantified via enzyme immunoassay (IBL-International, Männedorf, Switzerland). Plasma FFA concentrations were determined by an enzymatic colorimetric method (Wako Diagnostics, Tokyo, Japan).

*Immunoblotting* Approximately 30 mg of muscle was homogenized in ice-cold buffer as previously described (27). Lysates were centrifuged at 12,000 g for 30 min at 4 °C and the supernatant was transferred to a sterile microcentrifuge tube and aliquoted to measure protein concentration using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Lysate was then re-suspended in 4X Laemmli sample buffer with 40 µg of protein loaded onto 4–

20% Mini-PROTEAN TGX Stain-Free™ Gels (BioRad Laboratories, Gladesville, Australia). Post electrophoresis, gels were activated on a Chemidoc according to the manufacturer's instructions (BioRad Laboratories, Gladesville, Australia) and then transferred to polyvinylidine fluoride (PVDF) membranes. After transfer, a stain-free image of the PVDF membranes for total protein normalization was obtained before membranes were rinsed briefly in distilled water and blocked with 5% non-fat milk, washed with 10 mM of Tris–HCl, 100 mM of NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1000) overnight at 4 °C. Membranes were then incubated with secondary antibody (1:2000), and proteins were detected via enhanced chemiluminescence (Thermo Fisher, Scoresby, Australia) and quantified by densitometry (ChemiDoc™ XRS+ System; BioRad Laboratories, California, USA). Exercise and Non-Exercise pre- and post-samples were run on the same gel. Primary antibodies used were phospho- 4E-BP1Thr37/46 (#2855), mTORSer2448 (#2971), p70S6KThr389 (#9205), AMPKαThr172 (#2531) and total 4E-BP1 (#9644), mTOR (#2972), p70S6K (#9202) and AMPKα (#2532). All antibodies were purchased from Cell Signaling Technology (Danvers, USA). Volume density of each target phospho protein band was normalized to its respective total protein content, while the total protein band was normalised to the total protein loaded into each lane using stain-free technology (28), with data expressed in arbitrary units.

*RNA Extraction, Quantification, Reverse Transcription and Real-Time PCR* Skeletal muscle tissue RNA extraction was performed using a TRIzol-based kit according to the manufacturer's protocol (Invitrogen, Melbourne, Australia). In brief, approximately 20 mg of frozen skeletal muscle was homogenized in TRIzol with chloroform added to form an aqueous RNA phase. This RNA phase was then precipitated by mixing with isopropanol alcohol and the resulting pellet was washed and re-suspended in 50 μL of RNase-free water. Extracted RNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Scoresby, VIC, Australia). Reverse transcription and real-time Polymerase Chain Reaction (RT-PCR) was performed as previously described (29). In brief, first-strand complementary DNA (cDNA) synthesis was performed using commercially available TaqMan Reverse Transcription Reagents (Invitrogen, Melbourne, Australia). Quantification of mRNA in duplicate was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio Rad, California, USA). TaqMan-FAM-labelled primer/probes for Atrogin-1 (Hs01041408_m1), Myostatin (Hs00976237_m1), Muscle Ring Finger-1 (MuRF-1; Hs00822397_m1), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

(PGC-1α; Hs01016719_m1), Pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4; Hs01037712_m1), IL-6 (Hs00174131_m1), G protein-coupled receptor 56 (GPR56; Hs00938474_m1), and Fatty Acid Translocase Cluster of Differentiation 36 (FAT/CD36; Hs00354519_m1) were used in a final reaction volume of 20 µl. 18S ribosomal RNA (Cat. No. 4333760T) was used to normalize threshold cycle (CT) values and was stably expressed between post-exercise and between the different exercise conditions (data not shown). The relative amounts of mRNAs were calculated using the relative quantification (ΔΔCT) method (30).

*Statistical analysis*

For all DPP analyses, baseline differences between the independent groups were investigated by one-way ANOVA of Pre samples taken prior to deuterium oxide administration using *R* software for statistical computing. Within subject differences that occurred among Pre, Day 3, Day 6 and Day 9 samples were investigated using repeated measures ANOVA and separate tests were performed to investigate differences in either REX or HFLC groups. To control the false discovery rate (FDR), P-value distributions were used to calculate q-values and a criterion FDR of <1% was set. This statistical approach considers the biological variation across each protein and is, therefore, more sophisticated than arbitrarily implementing a threshold based on fold-change. Statistics for remaining analyses were performed using SigmaPlot (Version 12, Systat Software). Normal distribution and equal variance of the data were tested using Shapiro-Wilk and F tests, respectively. Two-way ANOVA followed by Student Newman Kuel's post-hoc tests were performed to determine differences between REX and HFLC groups, and time (Pre, D3, D6, and Post). All data in text and figures are presented as mean ± SEM with P values <0.05 indicating statistical significance.

## Results

*Effects of High Fat Low Carbohydrate Diet and Resistance Exercise on Glucose Metabolism, Free Fatty Acids and Plasma Markers of Inflammation and Appetite Control*

Consumption of HFLC diet did not result in differences in plasma insulin or glucose in response to an OGTT in either group (Figure 2A, B) although REX tended to lower glucose area under the curve between D0 and D9 (Figure 2C, P = 0.150). The HFLC diet increased fasting plasma non-esterified free fatty acids (FFA) in both groups, measured on the four mornings muscle biopsies were taken (P<0.05, Figure 3A).

There were main effects for time for IL-6 and TNF-α ($P<0.05$; Figure 3). IL-6 increased ~390% at Day 6 compared to Post in participants that performed REX ($P<0.05$). Similarly, TNF-α was highest at Day 6 in both groups and was ~205% and ~140% above Pre in REX and HFLC groups, respectively ($P<0.05$, Figure 3). There were non-significant increases in C-reactive protein, Pre-to-Post intervention (Figure 3). We also investigated the effects of our HFLC diet on the appetite and energy balance regulating hormones ghrelin and leptin. We observed non-significant decreases in these markers following our dietary intervention. There were also no changes in the concentrations of adiponectin or plasminogen activator inhibitor type 1 (PAI-1), circulating factors previously associated with insulin resistance (31, 32), between the REX and HFLC groups or throughout the dietary intervention (Figure 3).

*Dynamic proteome profiling of human muscle proteins*

On average, the molar per-cent enrichment (MPE) of $D_2O$ in body water increased at a rate of 0.135 ± 0.005 % per day and reached a peak of 2.14 ± 0.08 % on day 9. Label-free proteome profiling was conducted on proteins that had one or more unique peptides that were measured in all 16 participants at each of the four experiment time points. In the myofibrillar fraction, 38 proteins were analysed and included each of the major components of the muscle thick and thin myo-filaments. Analysis of the sarcoplasmic fraction encompassed 88 proteins with the majority of these having biological functions associated with the generation of precursor metabolites and belong to KEGG pathways including glycolysis/glycogenolysis, the tricarboxylic acid cycle and pyruvate metabolism.

The rate constant (k) of synthesis of each protein was calculated by fitting mass isotopomer data to a non-linear model (Figure 4) described previously in (19). After filtering to remove peptides with relatively poor fitting data ($R^2$ <0.99), 31 of the 38 myofibrillar proteins and 60 of the 88 sarcoplasmic proteins were retained (Supplementary tables report raw (S1 and S2) and summary (S3) mass spectrometry data). Figure 5 displays the rank order of synthesis rates for the 91 proteins investigated in the muscle of both groups of participants. Pyruvate dehydrogenase E1 component subunit beta (ODPB) had the lowest synthesis rate (0.08 ± 0.08 % per day), whereas carbonic anhydrase 3 (CAH3) exhibited the greatest synthesis rate (23.8 ± 5.8 % per day). The median and inter-quartile range (IQR) synthesis rate was 2.29 % per day (IQR 1.4 - 3.2 %/d) in the myofibrillar fraction and 3.1 % per day (IQR 0.9 – 3.9 %/d) in sarcoplasmic fraction. There was no difference ($P=0.317$) in the rate of synthesis of proteins analysed in the myofibrillar versus sarcoplasmic fractions. There was no association (ρ -0.08,

P=0.3925) between the rate of synthesis of a protein and its relative abundance in either the myofibrillar or sarcoplasmic fractions.

*The effect of High Fat Low Carbohydrate Diet and Resistance Exercise on muscle protein abundances*

Prior to the diet and exercise intervention there were no differences in muscle protein abundance between groups. The relative proportions of myosin heavy chain (MyHC) isoforms, which are commonly used to phenotype skeletal muscle, were 60 % MyHC 1, 31 % MyHC 2a and 9 % MyHC 2x in REX participants and were similar to participants in the HFLC group (63 % MyHC 1, 28 % MyHC 2a and 9 % MyHC 2x). Within-subject analysis of changes in protein abundance over the duration of the study period did not reveal any significant changes in protein abundance in the muscle of HFLC participants. In contrast, 17 proteins exhibited significant differences in abundance between baseline (D0) and D9 in the muscle of the REX group. With the exception of troponin C, the changes in protein abundance were reported from analysis of the sarcoplasmic fraction. Figure 6 presents a volcano plot of protein abundance data from REX participants and displays the fold change in protein abundance between D0 and D9 of the intervention. In total, 11 proteins increased and 6 decreased in abundance in response to the resistance exercise training and diet intervention.

*The effect of High Fat Low Carbohydrate Diet and Resistance Exercise on the synthesis of muscle proteins*

The average rate of synthesis of myofibrillar proteins was 1.58 ± 0.08 % per day in participants that performed REX. This synthesis response was significantly greater than the 1.09 ± 0.06 % per day in the muscle from HFLC only participants ($P< 0.001$, Figure 7). This effect of REX was not detected in the sarcoplasmic fraction where the average synthesis rate was similar between groups (REX = 2.15 ± 0.15 % /d, HFLC = 2.05 ± 0.17 % /d). When considered on a protein-by-proteins basis, 13 proteins (7 myofibrillar and 7 sarcoplasmic) had significantly greater rates of synthesis in the REX group ($P<0.05$, Figure 8).

*Protein responses to High Fat Low Carbohydrate Diet and Resistance Exercise*

Figure 9 summarises data from the REX group comparing within subject changes in protein abundance with between group analysis of differences in protein synthesis rate against the HFLC group and illustrates the variety of different responses of muscle proteins to resistance exercise. Proteins positioned in the upper left quadrant were less abundant after REX but

exhibited a greater rate of synthesis in exercised versus non-exercised muscle. Conversely, the abundance of proteins in the lower right quadrant increased in response to resistance exercise but their rate of synthesis was less than that measured in non-exercised muscle that did not exhibit any change in protein abundance.

Table 2 categorises exercise responsive proteins (categories i-vi) according to the pattern of the response observed in the REX group. The most common response to exercise was an increase in turnover rate without any significant change in abundance, and proteins that exhibited this response were mostly of myofibrillar origin (10 proteins). The second and third most numerous groups of proteins exhibited significant changes in abundance that were primarily attributable to differences in breakdown rate (i.e. protein abundance changed but there was no significant difference in synthesis rate). Proteins that increased in abundance (Table 2 category ii) included cytoskeletal binding proteins (ACTS, FLNC and PROF1) and cytoplasmic or membrane-bound vesicle proteins (ALBU, PRDX6, TRFE) that have anti-oxidant (PRDX6, ALBU) functions, whereas glycolytic enzymes were common amongst proteins that decreased in abundance (Table 2 category iii). Two other glycolytic enzymes exhibited unique responses including beta enolase, which was significantly less abundant after exercise training despite its rate of synthesis being significantly greater in exercised versus non-exercise trained muscle. Conversely, aldolase A did not change in abundance in REX muscle but its rate of synthesis was significantly less than reported in the HFLC group.

*Modulation in gene and protein markers of translation initiation mitochondrial biogenesis, and muscle proteolysis following HFLC diet*

The expression of the ubiquitin E3 ligases Muscle-specific RING Finger protein1 (MuRF1) and Muscle Atrophy F-box (MAFbx/atrogin-1) decreased ~160% post-intervention in the REX group only, resulting in a ~115-230% differential expression between groups post-intervention ($P<0.05$; Figure 9). Myostatin decreased by 140% post-intervention in the REX cohort and compared to the HFLC (~200%, $P<0.05$; Figure 10). REX also attenuated PPARδ mRNA expression compared to the non-exercise group by ~40% post-intervention (Figure 9), while the transcriptional co-activator PGC-1α decreased by ~130% in both groups post-intervention (Figure 9). There were no changes in FAT/CD36, GPR56, PDK4 or IL-6 mRNA expression (Figure 9). Western blot analyses on key mTOR related signalling substrates that regulate translation initiation processes revealed a ~50% increase in total mTOR abundance in REX compared to HFLC post-intervention ($P<0.05$; Figure 11). The phosphorylation state

or total amount of p70S6K, 4E-BP1 and AMPK were unaltered between groups or post-intervention (Figure 10).

## Discussion

In an effort to find therapeutic strategies to combat conditions such as sarcopenia, the anabolic response of muscle to resistance exercise and nutrition has been a longstanding focus of many clinical investigations. Previous application of peptide mass spectrometry and deuterium oxide has enabled measurement of synthesis rates for numerous proteins in human muscle (20). For the first time we report fully integrated proteomic profiling in association with deuterium labelling to simultaneously measure the rate of synthesis, net abundance and rate of breakdown of human muscle proteins in response to a diet-exercise intervention. Muscle anabolism induced by resistance exercise is typically regarded as being driven by synthetic processes, but using dynamic proteome profiling (DPP), we provide novel data to show that exercise-induced changes in muscle protein occur through several different patterns of response involving modulation of both synthetic and degradative processes. Such multiplicity in the adaptive response of the proteome (Figure 8) has not previously been captured and is a unique aspect of the current investigation.

New techniques have recently been developed incorporating $D_2O$ labelling with proteomic analyses including peptide mass spectrometry, which measure the rate constant (k) for synthesis on a protein-by-protein basis in the muscle of animals (33-36) and humans (20). Collectively, these works and our current data (Figure 5) demonstrate that there is a broad range of different synthesis rates of muscle proteins. These results highlight the advantages of interrogation of protein-specific synthetic responses (as opposed to the average gross synthesis of mixed proteins from whole muscle or muscle fractions) to discover novel therapeutic targets and provide mechanistic insight into protein metabolism. Moreover, results from these studies that show that protein synthesis rates differ, providing evidence for the selective breakdown of proteins at different rates in order to maintain the same relative abundance of proteins in tissue (i.e. maintenance of muscle mass). Chronic resistance training (i.e., 16 weeks) is associated with selective changes in protein abundance, such as a shift toward a greater proportion of the type IIa myosin heavy chain isoform (37). Resistance exercise increases the synthesis of mixed myosin heavy chains (38) but our data (Figures 4 and 8) is the first to shown this effect is confined to the fast-twitch IIa (MYH2). This ability

553 to measure the synthesis of key proteins such as MYH2 will be important to the future study
554 of the effects of resistance training in older adults with muscle wasting conditions.

555 In the current study, we reasoned that, when investigated at a protein-specific level, the
556 average rate of breakdown can be estimated from the difference between its measured change
557 in relative abundance and its rate of synthesis over that time period. That is, because the
558 synthesis, abundance and degradation of the protein are intimately connected then any change
559 in abundance that is not explained by the measured rate of synthesis during that period may
560 be a function of the rate of breakdown of that protein. Eleven proteins (Table 2) exhibited
561 negative values for the calculated rate of breakdown (i.e. the measured increase in protein
562 abundance was greater than the accumulative increase predicted from the measured synthesis
563 rate). While this may be attributable to technical errors associated with the measurement of
564 protein abundance and/or synthesis rate, it may also reflect secretion and delivery to muscle
565 of pre-existing protein, which would have the effect of increasing protein abundance to a
566 greater extent than predicted by synthesis alone. ALBU and TRFE each exhibited 'negative'
567 breakdown rates and, while abundant in muscle (15), these proteins are predominantly
568 expressed in the liver, secreted into the plasma and become resident in the muscle interstitial
569 space, which would have the effect of concentrating their abundance in a process distinct
570 from the synthesis process capture by deuterium oxide labelling. Similarly, the increases in
571 ACTS and TNNC2 could be explained by the translocation of soluble, pre-myofibril
572 complexes from the sarcoplasmic fraction and incorporation into mature myofibrils through
573 the process of myofibril assembly (39).

574 The potential involvement of myofibril assembly brings context to other findings from our
575 DPP data. Pre-myofibrils contain thin filament proteins (e.g. actin, troponin and
576 tropomyosin), costameric proteins (e.g. desmin and filamin) and z-band/z-body proteins (e.g.
577 alpha-actinin) that were responsive to resistance exercise (Table 2). Desmin has largely been
578 associated with lateral force transmission from contracting sarcomeres to the muscle exterior
579 (40) and is already known to increase in human muscle after resistance training (41). Our
580 findings raise the question whether part of this response is associated with myofibril
581 assembly, often an understudied area in the context of exercise adaptation. We also report
582 increases in the abundance of ankyrin repeat domain protein 2 (ANKR2) and 27 kDa heat
583 shock protein (HSPB1) in the sarcoplasmic fraction, and a greater turnover of alpha B-
584 crystallin (CRYAB) specifically in the myofibrillar fraction of exercised muscle. These

findings are consistent with earlier work (42, 43) and the responses observed for these proteins may be necessary to stabilise cytoskeleton and myofibril structures or prevent protein denaturation (44). Similarly Alpha B-crystallin translocates to and stabilises myofibrils in response to contractile stress (45) and this event is evidenced in our DPP data by the calculated 'negative' breakdown rate for CRYAB.

The greater (1.58 %/d) synthesis of myofibrillar proteins in exercised muscle (Figure 6) matches the 1.6 %/d rate reported by Brook et al (13) during the first three weeks of a similar resistance training stimulus. A potential limitation of our experimental design is that we did not include a healthy 'control' diet so we cannot compare the effect of a HFLC diet on muscle protein synthesis to other dietary interventions. Nevertheless, the average synthesis rate of myofibrillar proteins (1.09 %/d) in HFLC participants is less than reported in untrained muscle (1.35 %/d) by Brook et al (13). Of note was that the HFLC diet alone did not alter the abundance of muscle proteins suggesting there was little effect on muscle protein synthesis response. When combined with resistance exercise there was a decrease in the abundance of four glycolytic enzymes (Table 2, categories iii and v) and a lower synthesis rate for aldolase A. This contrasts with the increase in glycolytic metabolism that has previously been associated with resistance training (46, 47) although we also report two glycolytic enzymes (Table 2, category i) exhibited greater turnover in exercised muscle. This response may be associated with maintaining enzyme pool efficiency (19, 48) and a similar response has been reported previously in mouse heart during isoprenaline-induced cardio-toxicity (37).

MEK-ERK signalling has previously been associated with anabolic responses to resistance exercise (49, 50) and may have contributed to the differences between HFLC and REX groups. Degradation of phosphatidylethanolamine-binding protein 1 (PEBP1; also known as RAF kinase inhibitor protein RKIP) was greater in exercised muscle and resulted in significantly less RKIP abundance, which may enhance signalling through the MEK-ERK pathway (51). RKIP is also a component in the diacylglycerol (DAG)-sensitive PKC pathway that increases IL-6 expression in response to saturated fatty acids (52) and we observed greater IL-6 mRNA expression and plasma concentration of IL-6 in resistance-exercised individuals. New data from the model organism *C. Elegans* also implicates RKIP in protein breakdown (53) and RKIP/PEBP1 was reported to be an inhibitor of starvation-induced autophagy in mammalian cells (54). Here, mRNA expression of ubiquitin E3 ligases

(Atrogin-1 and MuRF-1) was lower in exercised muscle, which may indicate the ubiquitin-proteasomal system is not the primary mechanism for the observed greater breakdown of selected proteins (Table 2, categories i, ii and iii). Other processes that could be responsible for the breakdown of these proteins include chaperone-assisted selective autophagy (CASA). Filamin-C (FLNC), which is an actin-binding protein that may be involved in mechano-transduction and intracellular signalling (55) and Ulbricht and colleagues recently demonstrated increased FLNC mRNA and protein levels after maximal eccentric exercise in human skeletal muscle (56). As filamin-C is thought to be damaged during eccentric muscle contraction it may require removal by autophagy in exercised muscle (57). CASA is implicated in these responses and inhibition of CASA may also be responsible, in part, for the increased abundance (Table 2 category ii) detected with DPP and RKIP.

In conclusion, the response of human muscle protein turnover to resistance exercise training differs on a protein-by-protein basis (Table 2). Of the proteins that were altered by the exercise stimulus, the most common response was an increase in turnover (i.e. the increase in synthesis rate was matched by an increase in the rate of breakdown resulting in no measurable change in protein abundance). We report that changes in protein abundance in response to resistance exercise are underpinned by regulation of either synthetic or degradative processes, or a combination of both. While such concepts are not new, it has been challenging to collect empirical data to support this paradigm in human tissue. We show DPP can provide data on the synthesis, abundance and breakdown of individual proteins. As such, use of DPP in future studies has the potential to significantly advance knowledge of exercise-induced adaptations as well as patho-biochemical processes associated with chronic human muscle-wasting diseases and muscle loss with ageing. For example, the application of DPP in chronic interventions could uncover how the response of enhanced turnover reported here is converted into protein accretion and myofibre hypertrophy, and whether increases in synthesis continue are sustained (and for how long) or if the rate of breakdown subsides with longer period of resistance exercise training.

**Conflict of Interest Statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author contributions

DMC, JGB and JAH designed the study, DMC and WJS conducted the experiment, DMC, JGB and MAP performed the biochemical assays and data analyses, DMC, JGB and JAH wrote the manuscript and all authors approved the manuscript prior to submission.


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**Figure legends**

**Table 1**.

Baseline Characteristics of the participants in the REX and HFLC groups.

**Table 2**.

Pattern of muscle proteome responses of individual proteins observed in the REX group between and D0 and D9. For the purpose of comparison/ calculation, changes in abundance have been converted to rate of change in abundance.

**Figure 1**. $D_2O$-labeling protocol and peptide enrichment. (A) After 3 days of dietary control participants consumed a high-fat low-carbohydrate (HFLC) diet (8% Carbohydrate, 77% Fat, 15% Protein) and undertook three sessions of resistance exercise (REX), or consumed the HFLC with no exercise (HFLC). Participants ingested deuterium oxide (200 ml/ d) during a 9-day intervention period, venous blood samples were collected daily and samples of *vastus lateralis* were collected prior to (i.e. morning of day 0) and during (days 3, 6 and 9) the intervention. Blood and muscle samples were analysed by mass spectrometry to determine precursor enrichment and the rate of incorporation of deuterium in to newly synthesised protein, respectively.

**Figure 2**. The effects of high-fat low-carbohydrate diet and resistance exercise on blood glucose homeostasis. Differences in plasma insulin (A), glucose (B) and glucose area under the curve (C) in participants who performed resistance exercise (REX) or consumed a high fat low-carbohydrate (HFLC) diet only. Data are presented as mean ± SEM; n = 8 in REX/ n = 7 in HFLC, a = $P < 0.05$ to 'Rest' within group, d = $P < 0.05$ to '90 min' within group, e = $P < 0.05$ to '120 min' within group; 2-way ANOVA.

**Figure 3**. Metabolite and hormone responses to high-fat low-carbohydrate diet and resistance exercise. Differences in plasma concentrations of Free fatty acids (A), Adiponectin (B), PAI-1 (C), IL-6 (D), TNF-α, (E), Leptin (F) and C-reactive protein (G) and (H) Ghrelin. Data represent resistance trained (REX) participants who performed exercise and consumed a high fat low carbohydrate diet and control (HFLC) participants who consumed the high fat low carbohydrate diet only. Data are presented as mean ± SEM; n = 8 in REX/ n = 7 in HFLC, * = $P < 0.05$ between REX and HFLC, # = $P < 0.05$ interaction for time; 2-way ANOVA.

718 **Figure 4**. (A) Example mass spectra of peptide LAQESIMDIENEK ([M+2H] 2+ = 760.3837
719 m/z) from the type IIa isoform (MYH2) of myosin heavy chain. The relative abundances of
720 m0 (monoisotopic), m1, m2, m3 and m4 mass isotopomers at D0, D3, D6 and D9 is
721 presented. The incorporation of deuterium in to newly synthesised protein is evident in the
722 proportional increase in the 'heavy' isotopomers (m1, m2, m3 and m4) and relative decrease
723 in the fractional abundance of the monoisotopic peak (i.e. m0/Σmi). The mean ± SD and
724 coefficient of variation (%CV) is reported for exercise trained participants (n = 8). (B) The
725 rate constant of synthesis was calculated by fitting mass isotopomer data (m0/Σmi) to a non-
726 linear model of the rise in precursor enrichment and incorporation of deuterium in to the
727 product peptide, taking in to account the amino acid composition of the peptide. (C) The
728 fractional synthesis rate (k) of MYH2 in exercised muscle (0.0187 ± 0.0032) was
729 significantly (P=0.016) greater than control (0.0096 ± 0.0017) based on mass isotopomer
730 distribution analysis of 5 peptides investigated in n=8 participants per group.
731
732
733 **Figure 5**. Rank order of protein synthesis rates in human skeletal muscle. (A) The mean rate
734 constant (k) for the synthesis of each protein ranked from least to greatest from D0 to D9.
735 Data represent proteins analysed in the myofibrillar pellet (blue) and sarcoplasmic/ soluble
736 muscle fraction (orange) of both diet control (HFLC) and resistance exercised (REX)
737 participants. Labels indicate the UniProt knowledgebase identifiers for a sub-selection of
738 proteins. The size (radius) of each data point represents the mean normalised abundance of
739 each protein in baseline (day 0) samples. (B) Boxplots presenting the average (median and
740 interquartile range) rate constant of synthesis of proteins analysed in the myofibrillar pellet
741 and soluble muscle fraction from D0 to D9. Independent two-tailed t-test found no significant
742 (P=0.317) difference in the average rate of synthesis of proteins in myofibrillar (n=31) vs
743 sarcoplasmic (n=60) proteins.
744
745 **Figure 6**. The effects of resistance exercise on muscle protein abundance. Volcano plot
746 presenting pre- (day 0) versus post-intervention (day 9) changes (Fold change (Log2)) in
747 resistance trained (REX) participants. There were no significant differences in protein
748 abundance in the muscle of control participants who consumed the high-fat low-carbohydrate
749 (HFLC) diet only. P values were calculated from log-transformed data using within-subject
750 (repeated measures) one-way analysis of variance. Proteins that exhibited a statistically
751 significant ($p<0.05$) change in abundances are coloured red if the calculated false discovery

752 rate (FDR) is < 5 % or blue if FDR is > 5 % and less than 10 %. Labels represent the Uniport
753 Knowledgebase identifier of each of the statistically significant proteins.
754
755 **Figure 7**. The effect of three bouts of resistance exercise on the average synthesis rate of
756 human muscle proteins. Data are presented using violin plots overlaid with box plots that
757 represent the average (median and interquartile range) rate of synthesis of mixed myofibrillar
758 or soluble muscle proteins. Exercise participants performed resistance training (REX) and
759 consumed a high-fat low-carbohydrate diet, whereas control (HFLC) participants consumed
760 the high-fat low-carbohydrate diet only (n = 8/group). Two-tailed independent t-tests found
761 the synthesis rate of mixed myofibrillar proteins was significantly ($P<0.001$) greater in REX
762 compared to HFLC. There was no significant ($P=0.531$) difference in the rate of synthesis of
763 sarcoplasmic proteins between groups.
764
765 **Figure 8**. The effects of resistance exercise on the synthesis rate of muscle proteins in REX
766 participants only. Volcano plot presenting differences (Fold-difference (Log2)) in the rate of
767 synthesis of proteins between participants who performed resistance exercise (REX) and or
768 those who consumed a high-fat low-carbohydrate (HFLC) diet only. P values were calculated
769 from log-transformed data (n=8/ group) using between-subject one-way analysis of variance.
770 Proteins that exhibited a statistically significant ($p<0.05$) change in abundances are coloured
771 red if the calculated false discovery rate (FDR) is < 5 % or blue if FDR is >5 % and less than
772 10 %. Labels represent the Uniport Knowledgebase identifier of each of the statistically
773 significant proteins.
774
775 **Figure 9**. Dynamic proteome responses to resistance exercise in the myofibrillar (A) and
776 sarcoplasmic (B) fractions. Summary data presented are from resistance exercised (REX)
777 participants only. There were no changes in protein abundance in HFLC control participants
778 who consumed a high-fat low-carbohydrate diet only. Upper left quadrant = decrease in
779 abundance over the duration of the experiment but greater rate of synthesis compared to
780 HFLC participants. Upper right quadrant = proteins that increased in abundance in the muscle
781 of REX participants and protein synthesis rate was also greater in REX compared to HFLC.
782 Lower left quadrant = Decrease in abundance after REX while synthesis rate is also less than
783 HFLC participants. Lower right quadrant = increase in abundance after REX despite the
784 synthesis rate being less than HFLC participants.
785

**Figure 10**. Differences in mRNA expression of (A) Atrogin-1, (B) MuRF-1, (C) Myostatin, (D) FAT/CD36, (E) GPR56, (F) PDK4, (G) PGC1-α, (H) PPAR-δ and (I) IL-6. Data represent REX participants who performed resistance exercise and consumed a high fat low carbohydrate diet and HFLC participants who consumed the high fat low carbohydrate diet only (mean ± SEM; n = 6 in REX/ n = 5 in HFLC, * = $P < 0.05$ between REX and HFLC, a = $P < 0.05$ to 'Rest' within group; 2-way ANOVA).

**Figure 11**. Differences in phosphorylation status of (A) 4E-BP1Thr37/46, (C) AMP KinaseThr172, (E) mTORSer2448, (G) p70S6KThr389, and total abundance of (B) 4E-BP1, (D) AMPKα, (F) mTOR and (H) p70S6K. Data represent REX participants who performed resistance exercise and consumed a high fat low carbohydrate diet and HFLC participants who consumed the high fat low carbohydrate diet only (mean ± SEM; n = 5 in REX/ n = 4 in HFLC, * = $P < 0.05$ between REX and HFLC; 2-way ANOVA).

**Table 1.**

Baseline Characteristics of the participants

| | REX (n = 8) | HFLC (n = 8) |
|---|---|---|
| Age (y) | 36.9 ± 1.9 | 36.9 ± 2.1 |
| Body Mass (kg) | 88.8 ± 4.2 | 85.5 ± 3.4 |
| BMI (kg $m^{-2}$) | 26.9 ± 1.0 | 26.4 ± 1.1 |
| Fat Free Mass (kg) | 60.4 ± 2.0 | 57.8 ± 2.6 |
| Fat Mass (kg) | 24.9 ± 2.9 | 24.5 ± 2.1 |
| $VO_{2peak}$ (ml/kg/min) | 39.7 ± 1.9 | 36.5 ± 2.2 |
| $VO_{2peak}$ (L/min) | 3.5 ± 0.1 | 3.1 ± 0.2 |
| Leg Extension 1-RM (kg) | 79.8 ± 5.0 | 76.5 ± 6.6 |
| Leg Press 1-RM (kg) | 221.0 ± 6.0 | 217.6 ± 16.1 |

Values are given as mean ± SEM.

821 **Table 2**

| Protein identifier (UniProt ID) | Protein name | Abundance change (%/d) | Fractional synthesis rate (%/d) | Fractional breakdown rate (%/d) |
|---|---|---|---|---|
| **(i) Increased turnover, no change in abundance** | | | | |
| ACON | Aconitase | -3.34 | 3.46 | 6.8 |
| ACTN2 | Alpha-actinin 2 | +1.83 | 4.54 | 2.71 |
| CRYAB | Alpha B-crystallin | +3.89 | 4.38 | 0.49 |
| DESM | Desmin | -1.55 | 4.25 | 5.8 |
| LDHA | Lactate dehydrogenase | -3.34 | 9.63 | 12.97 |
| MLRS | Myosin regulatory light chain 2 | +2.82 | 1.25 | -1.57 |
| MYH2 | Myosin heavy chain 2 | -0.11 | 1.88 | 1.99 |
| MYL1 | Myosin light chain 1/3 | +1.55 | 2.53 | 0.98 |
| PGM1 | Phosphoglucomutase 1 | -1.45 | 1.09 | 2.54 |
| TPM2 | Tropomyosin beta | 2.65 | 0.93 | -1.72 |
| **(ii) Significant increase in abundance with no difference in synthesis rate** | | | | |
| ACTS | Actin, alpha skeletal muscle | +3.17 | 1.41 | -1.76 |
| ALBU | Serum albumin | +8.9 | 3.21 | -5.69 |
| ENOA | Alpha enolase | +2.2 | 3.62 | 1.42 |
| FLNC | Filamin C | +8.13 | 12.45 | 4.32 |

| | | | | |
|---|---|---|---|---|
| IGHG4 | Ig gamma-4 chain C region | +14.7 | 8.77 | -5.93 |
| PRDX6 | Peroxiredoxin-6 | +2.12 | 6.07 | 3.95 |
| PROF1 | Profilin-1 | +3.73 | 1.22 | -2.51 |
| TNNC2 | Troponin C | +5.29 | 1.52 | -3.77 |
| TRFE | Serotransferrin | +10.56 | 7.25 | -3.31 |
| **(iii) Significant decrease in abundance but no significant difference in synthesis** | | | | |
| AT2A1 | Sarcoplasmic/endoplasmic reticulum Ca2+ ATPase | -1.45 | 6.90 | 8.35 |
| G3P | Glyceraldehyde-3 phosphate dehydrogenase | -2.74 | 0.24 | 2.98 |
| PEBP1 | Phosphatidylethanolamine-binding protein 1 | -2.21 | 0.42 | 2.63 |
| PGAM2 | Phosphoglycerate mutase 2 | -3.56 | 5.35 | 8.91 |
| TPIS | Triosephosphate isomerase | -2.12 | 0.72 | 2.84 |
| **(iv) Significant increase in abundance and significantly greater synthesis rate** | | | | |
| HSPB1 | Heat shock protein beta-1 | +4.72 | 6.22 | 1.5 |
| ANKR2 | Ankyrin repeat domain-containing protein-2 | +4.43 | 6.17 | 1.74 |
| **(v) Significant decrease in abundance and significantly greater synthesis rate** | | | | |
| ENOB | Beta-enolase | -1.84 | 6.62 | 8.46 |

| | | | | |
|---|---|---|---|---|
| **(vi) No change in abundance but a significantly lesser synthesis rate** | | | | |
| ALDOA | Fructose-bisphosphate aldolase A | -1.36 | 4.63 | 5.99 |

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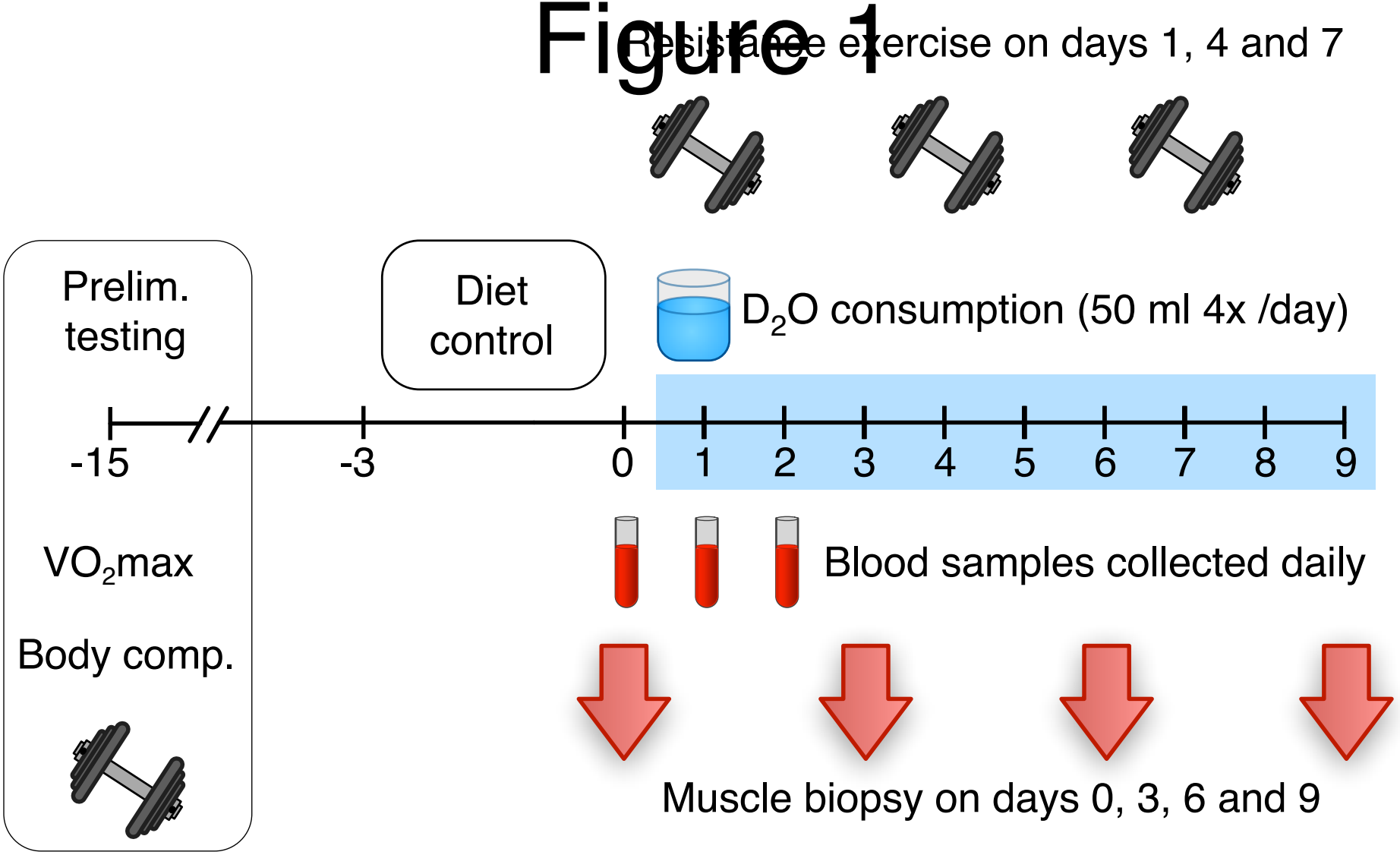
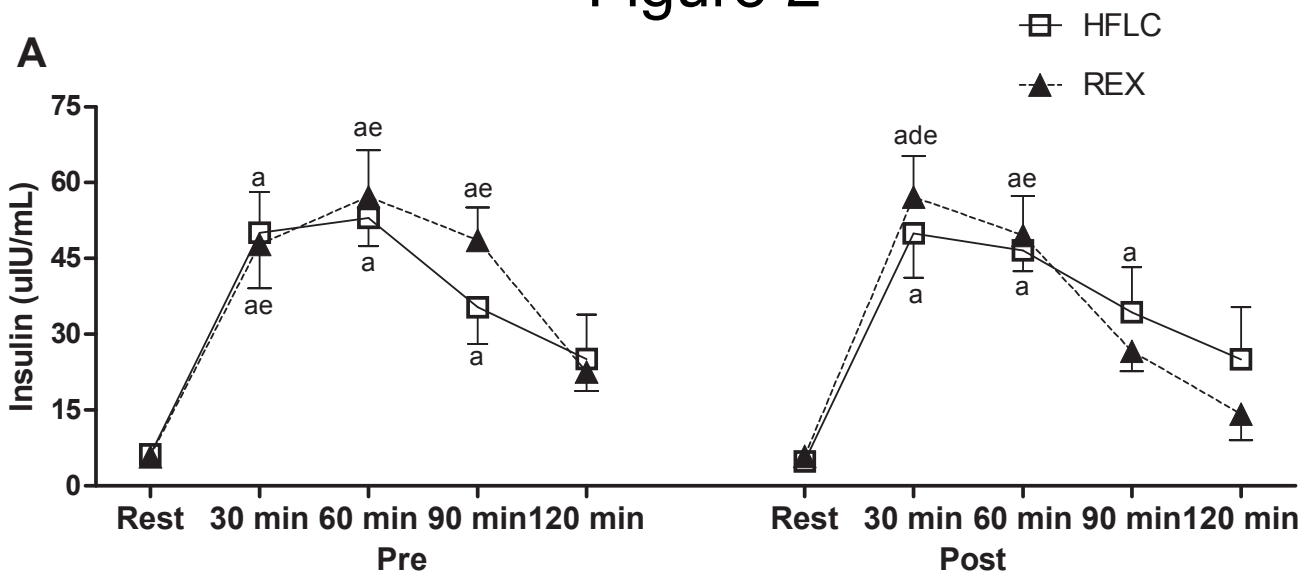
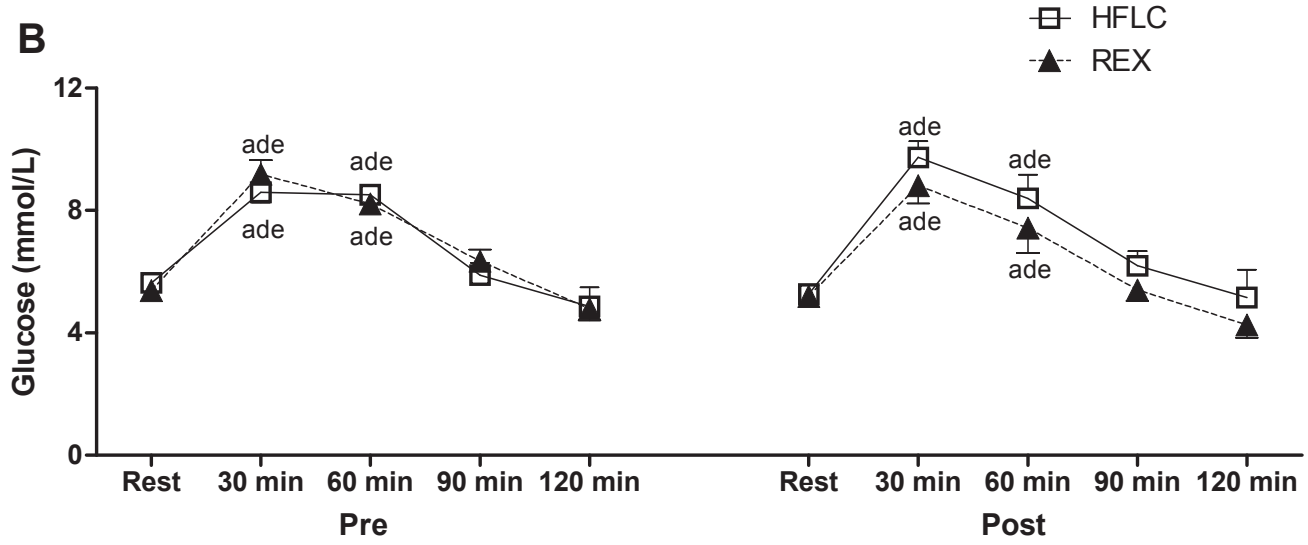
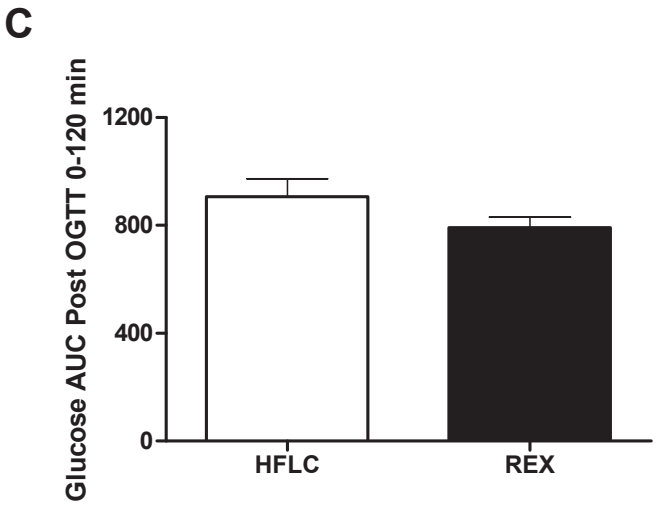
# Figure 1


Resistance exercise on days 1, 4 and 7
Prelim. testing
Diet control
$D_2O$ consumption (50 ml 4x /day)
-15
-3
0
1
2
3
4
5
6
7
8
9
$VO_2max$
Body comp.
Blood samples collected daily
Muscle biopsy on days 0, 3, 6 and 9

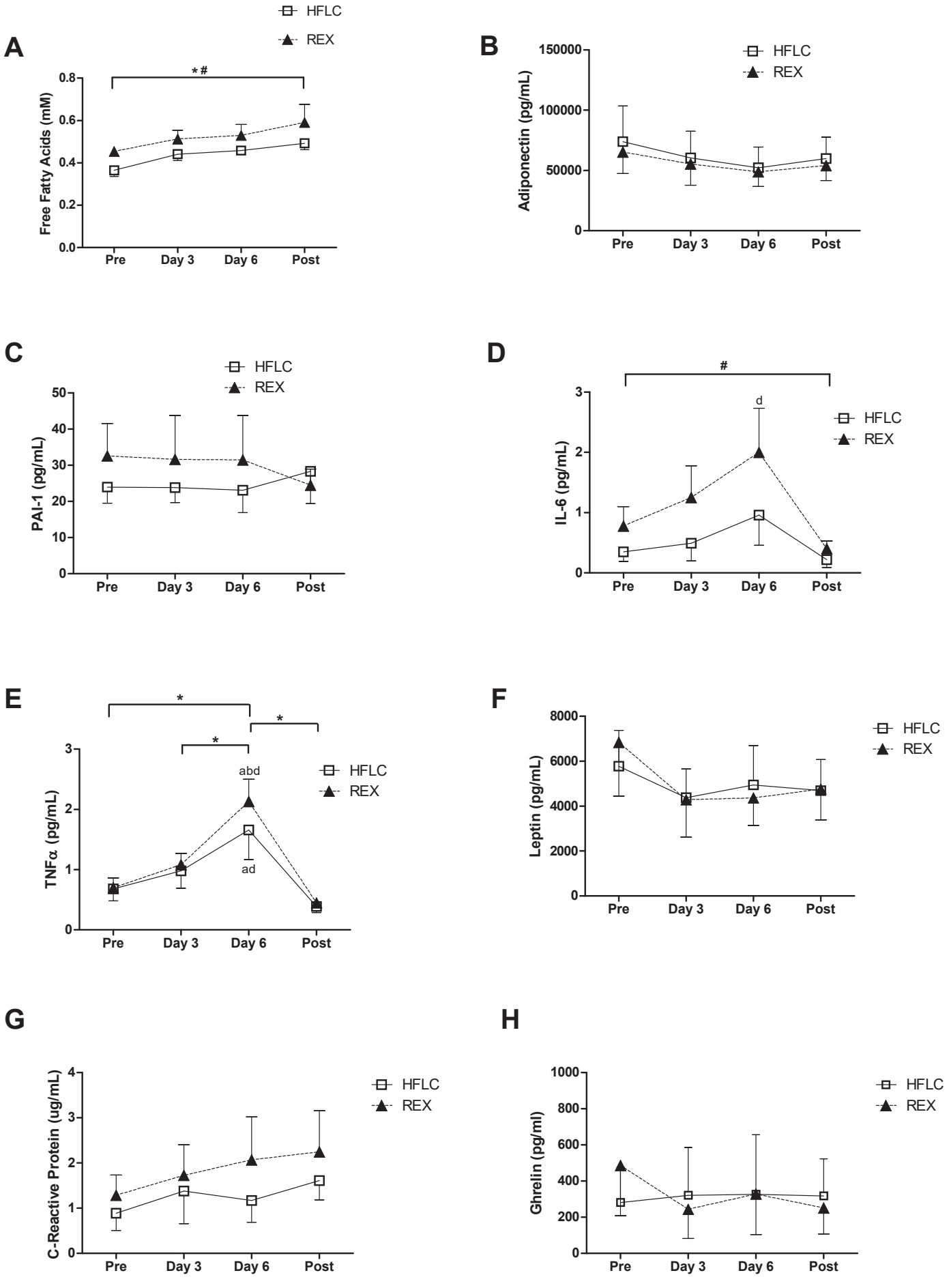
# Figure 2


A
HFLC
REX
Insulin (uIU/mL)
75
60
45
30
15
0
a
ae
ae
a
ae
a
ade
a
ae
a
a
Rest
30 min
60 min
90 min
120 min
Pre
Rest
30 min
60 min
90 min
120 min
Post

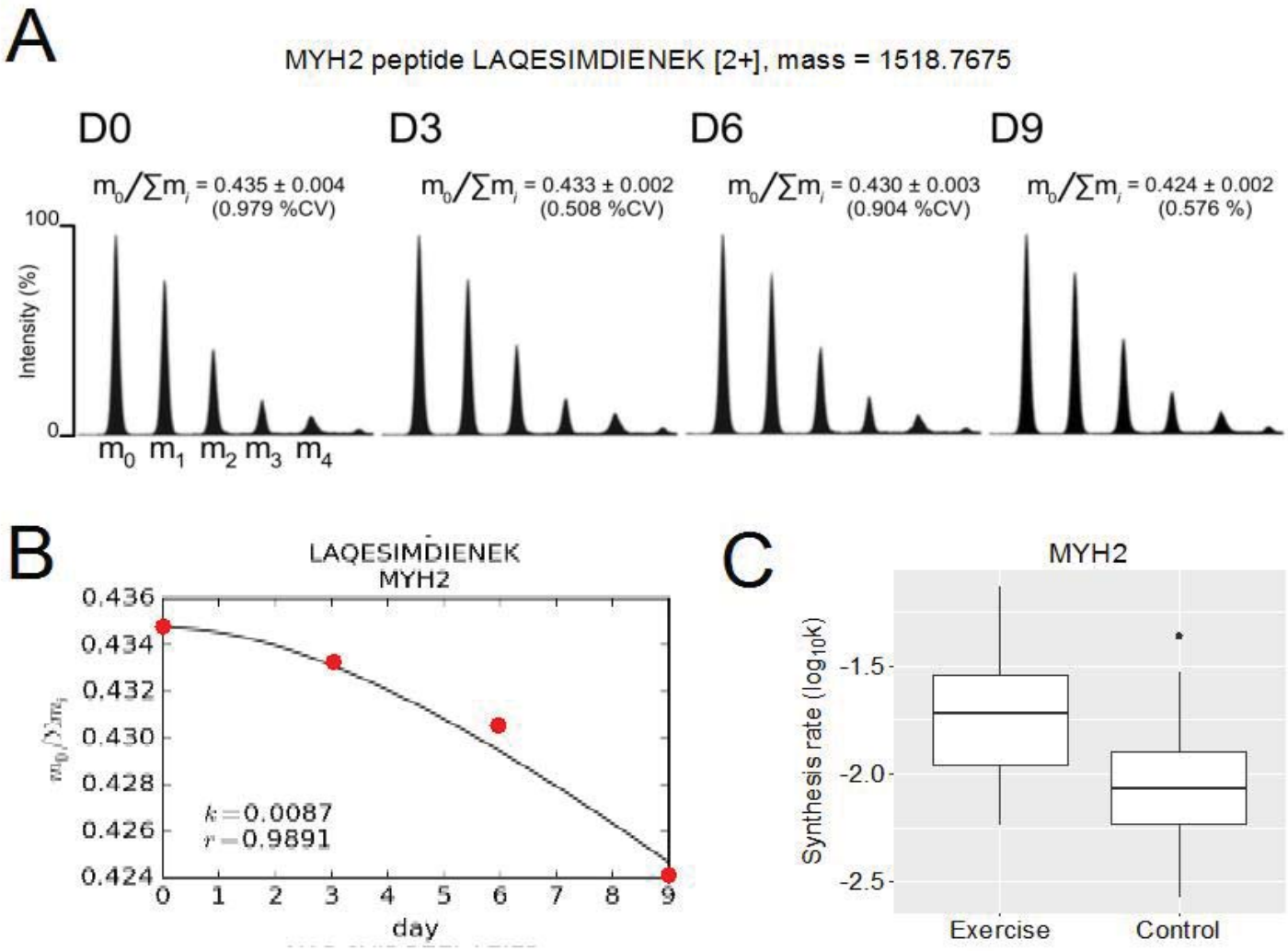

B
HFLC
REX
Glucose (mmol/L)
12
8
4
0
ade
ade
ade
ade
ade
ade
ade
ade
ade
Rest
30 min
60 min
90 min
120 min
Pre
Rest
30 min
60 min
90 min
120 min
Post


C
Glucose AUC Post OGTT 0-120 min
1200
800
400
0
HFLC
REX

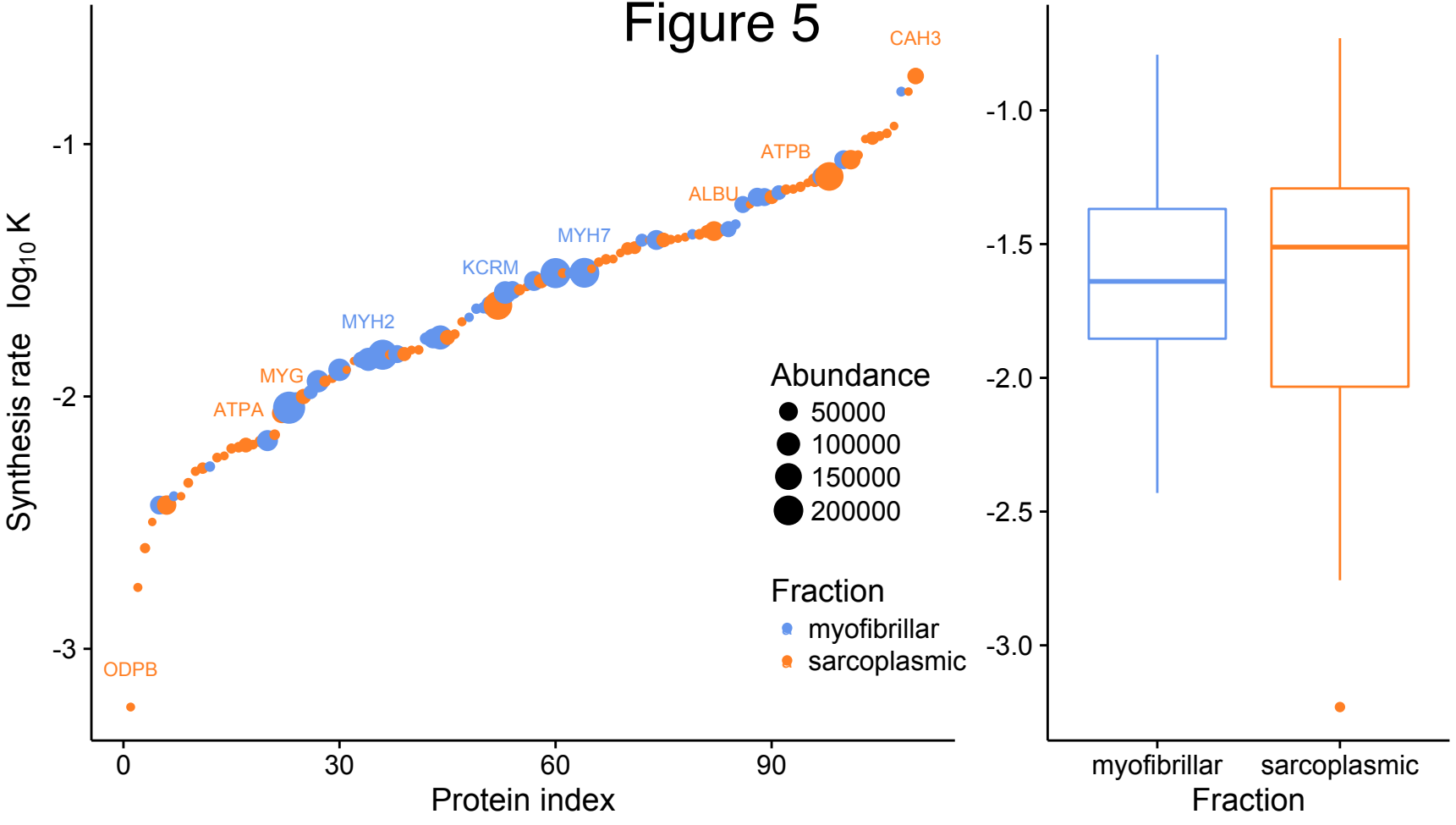
# Figure 3


A
HFLC
REX
*#
Free Fatty Acids (mM)
0.0
0.2
0.4
0.6
0.8
Pre
Day 3
Day 6
Post
B
HFLC
REX
Adiponectin (pg/mL)
0
50000
100000
150000
Pre
Day 3
Day 6
Post
C
HFLC
REX
PAI-1 (pg/mL)
0
10
20
30
40
50
Pre
Day 3
Day 6
Post
D
#
d
HFLC
REX
IL-6 (pg/mL)
0
1
2
3
Pre
Day 3
Day 6
Post
E
*
*
*
abd
ad
HFLC
REX
TNFα (pg/mL)
0
1
2
3
Pre
Day 3
Day 6
Post
F
HFLC
REX
Leptin (pg/mL)
0
2000
4000
6000
8000
Pre
Day 3
Day 6
Post
G
HFLC
REX
C-Reactive Protein (ug/mL)
0
1
2
3
4
Pre
Day 3
Day 6
Post
H
HFLC
REX
Ghrelin (pg/ml)
0
200
400
600
800
1000
Pre
Day 3
Day 6
Post

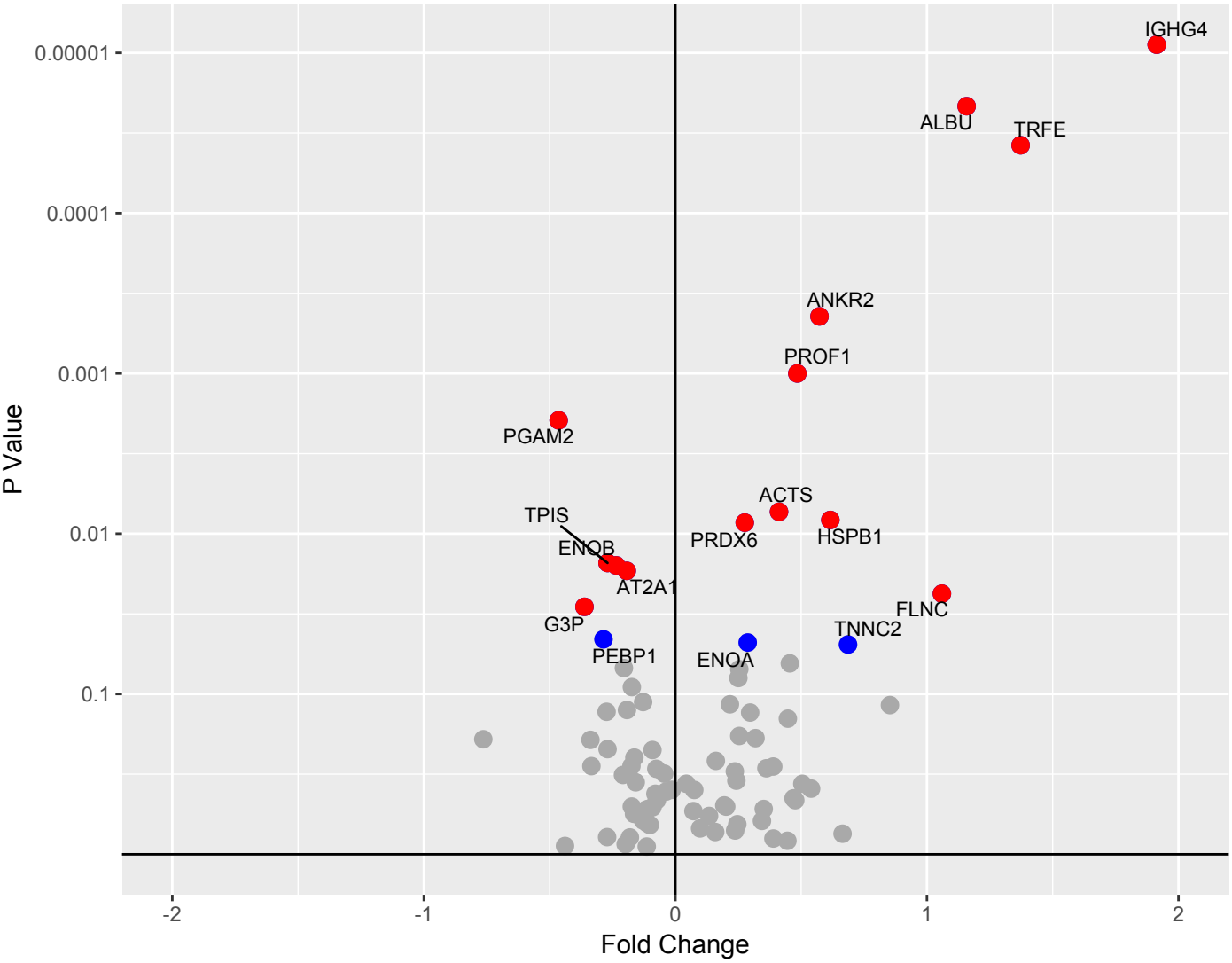
# Figure 4


A
MYH2 peptide LAQESIMDIENEK [2+], mass = 1518.7675
D0
D3
D6
D9
$m_0/\sum m_i$ = 0.435 ± 0.004 (0.979 %CV)
$m_0/\sum m_i$ = 0.433 ± 0.002 (0.508 %CV)
$m_0/\sum m_i$ = 0.430 ± 0.003 (0.904 %CV)
$m_0/\sum m_i$ = 0.424 ± 0.002 (0.576 %)
100
0
Intensity (%)
$m_0$ $m_1$ $m_2$ $m_3$ $m_4$
B
LAQESIMDIENEK
MYH2
0.436
0.434
0.432
0.430
0.428
0.426
0.424
$m_0/\sum m_i$
$k = 0.0087$
$r = 0.9891$
0 1 2 3 4 5 6 7 8 9
day
C
MYH2
Synthesis rate ($\log_{10}k$)
-1.5
-2.0
-2.5
Exercise
Control

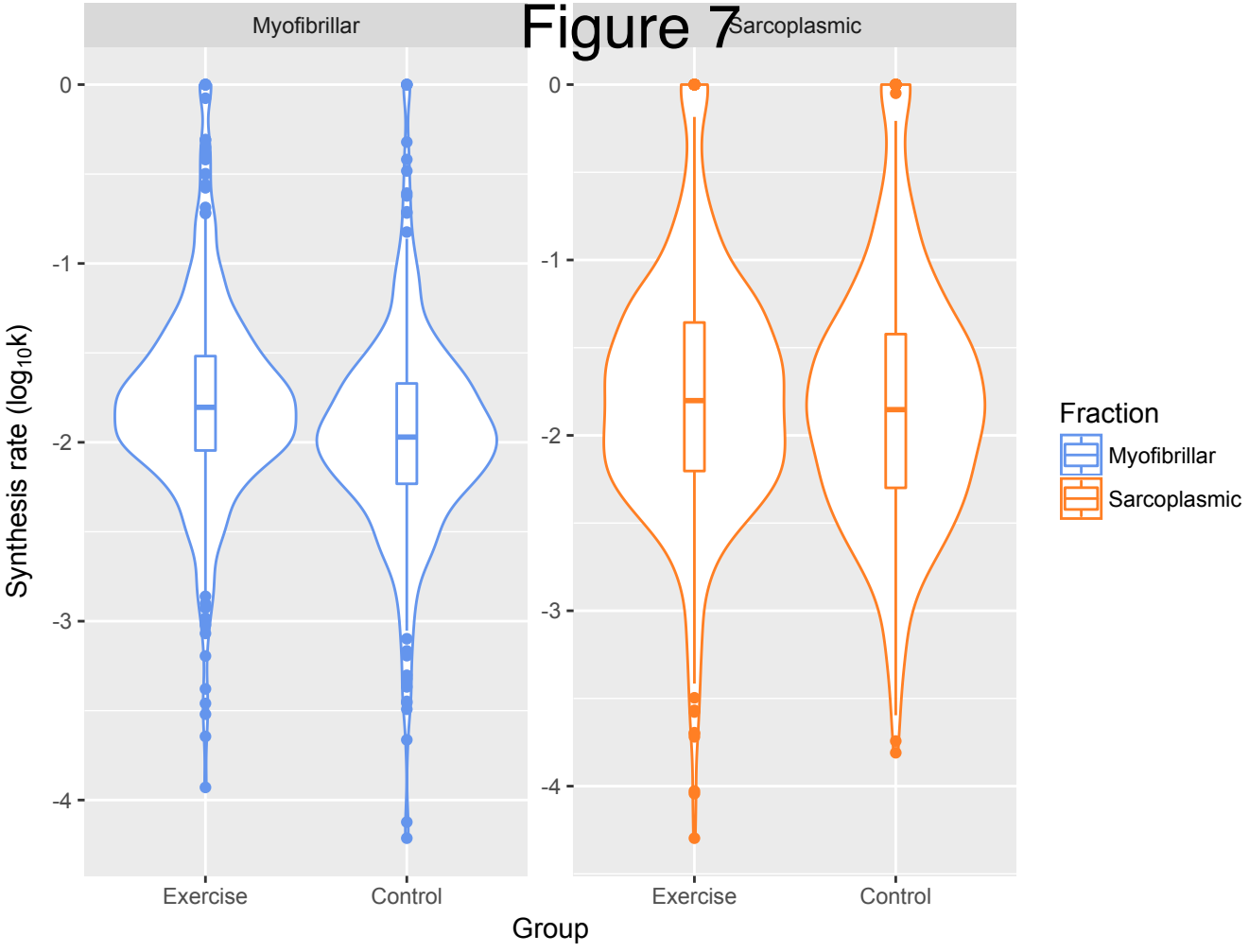
# Figure 5


Synthesis rate log10 K
-1
-2
-3
0
30
60
90
Protein index
CAH3
ATPB
ALBU
MYH7
KCRM
MYH2
MYG
ATPA
ODPB
Abundance
50000
100000
150000
200000
Fraction
myofibrillar
sarcoplasmic
-1.0
-1.5
-2.0
-2.5
-3.0
myofibrillar
sarcoplasmic
Fraction

# Figure 6


Volcano Plot
of protein abundance
IGHG4
ALBU
TRFE
ANKR2
PROF1
PGAM2
ACTS
TPIS
PRDX6
HSPB1
ENOB
AT2A1
FLNC
G3P
TNNC2
PEBP1
ENOA
0.00001
0.0001
0.001
0.01
0.1
P Value
-2
-1
0
1
2
Fold Change

# Figure 7


Myofibrillar
Sarcoplasmic
0
-1
-2
-3
-4
Synthesis rate (log10k)
Exercise
Control
Group
Fraction
Myofibrillar
Sarcoplasmic

# Figure 8

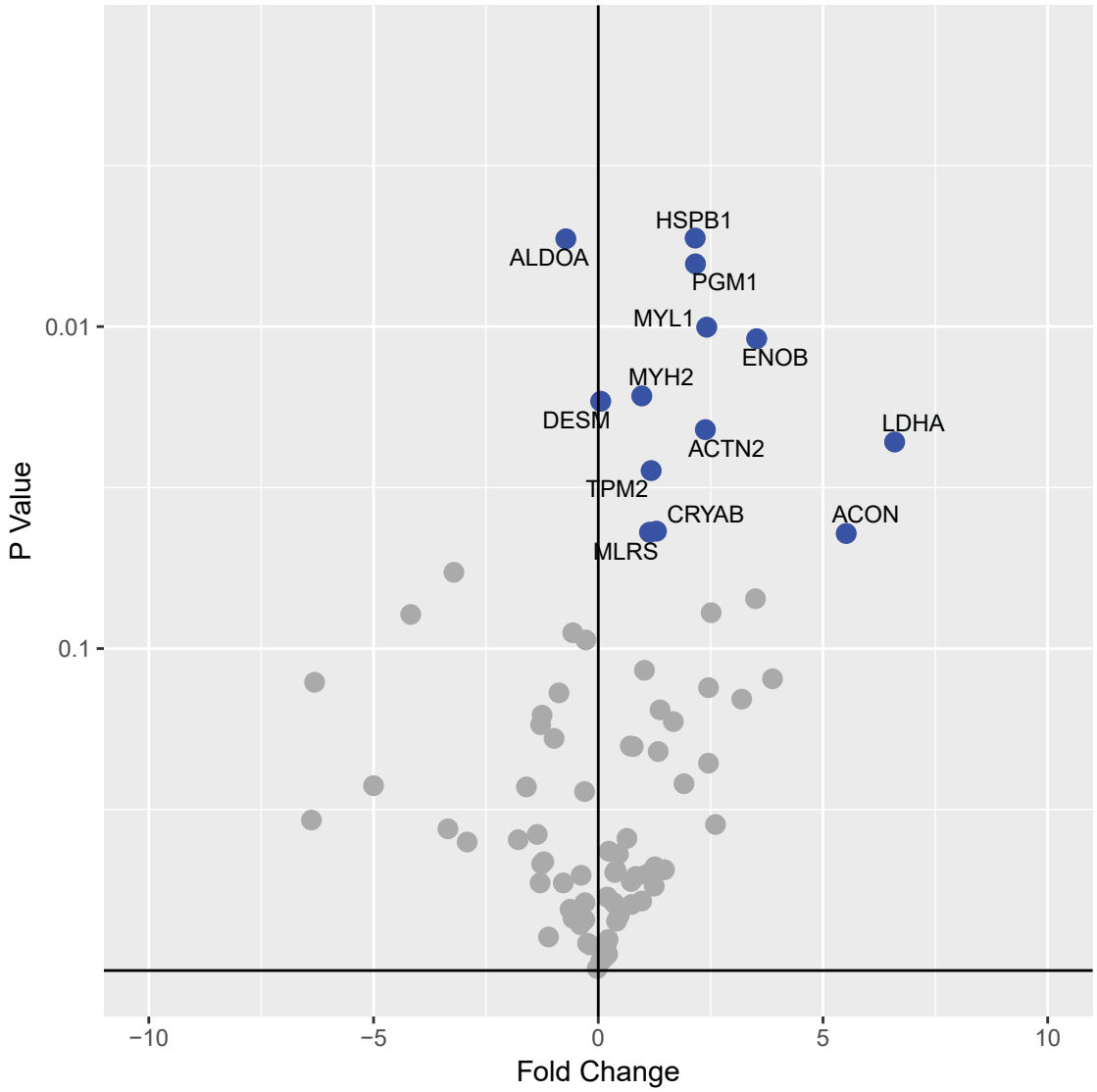
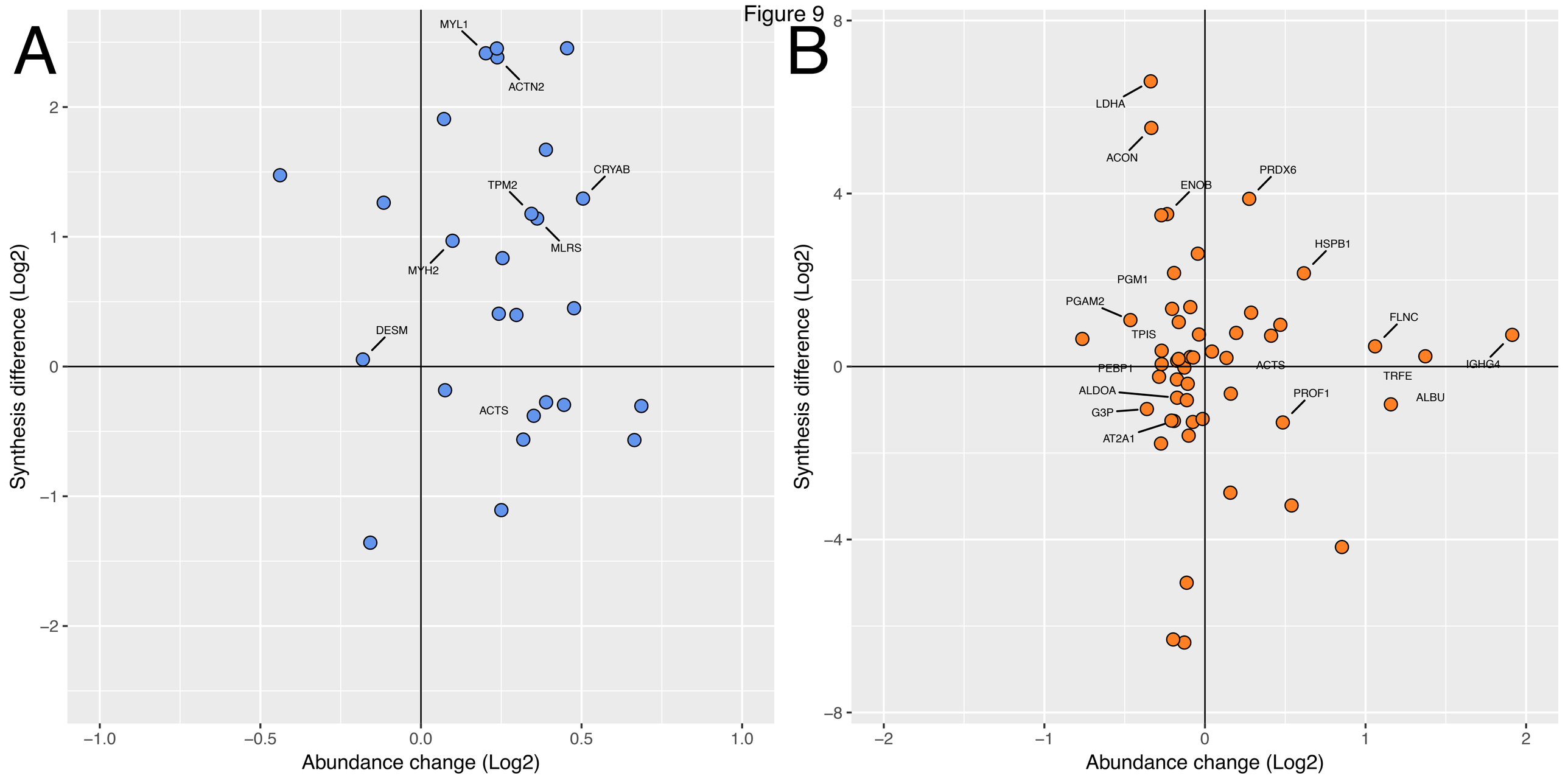
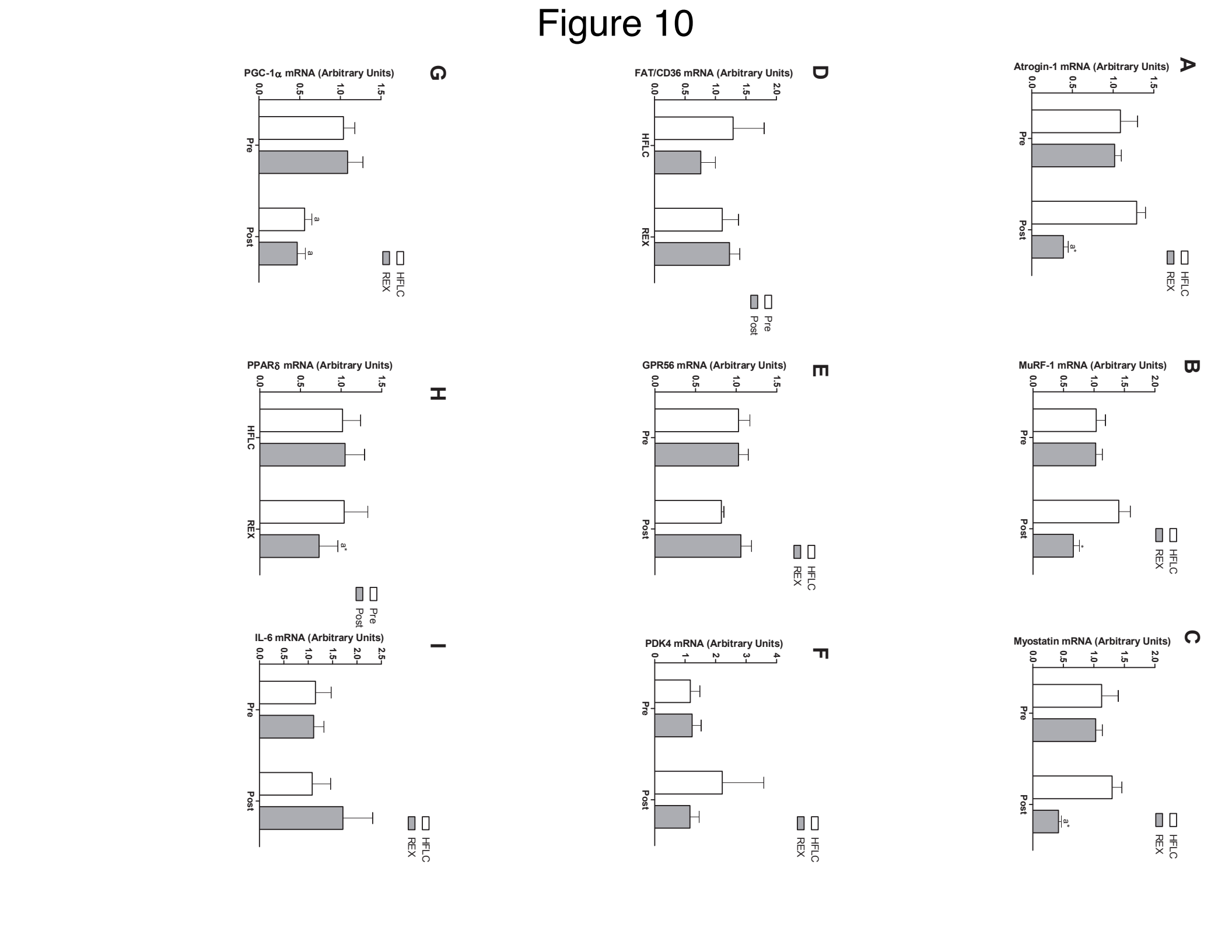

HSPB1
ALDOA
PGM1
MYL1
ENOB
MYH2
DESM
LDHA
ACTN2
TPM2
CRYAB
ACON
MLRS
0.01
0.1
P Value
−10
−5
0
5
10
Fold Change

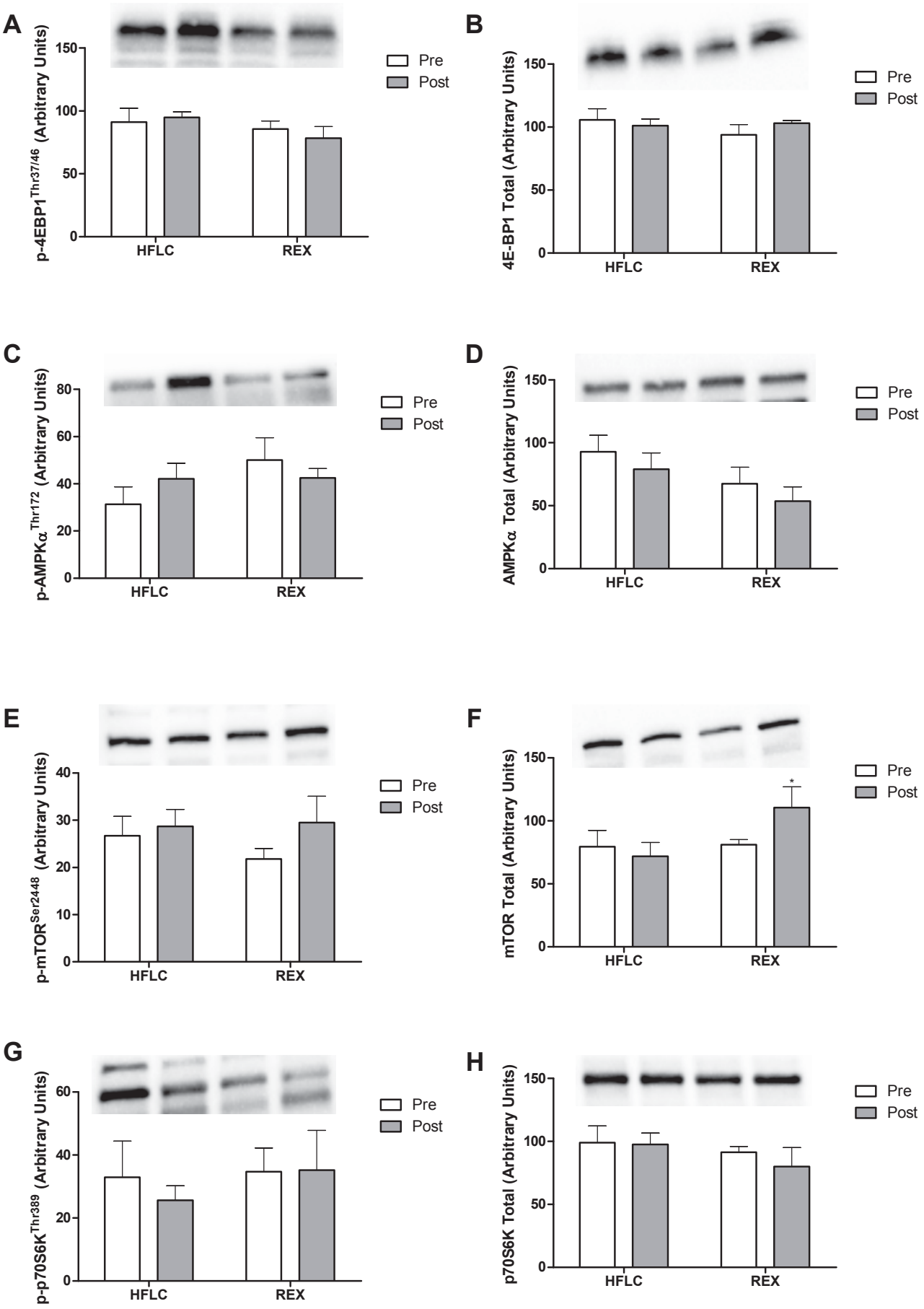
Figure 9


A
MYL1
ACTN2
CRYAB
TPM2
MLRS
MYH2
DESM
ACTS
2
1
0
−1
−2
Synthesis difference (Log2)
−1.0
−0.5
0.0
0.5
1.0
Abundance change (Log2)
B
LDHA
ACON
PRDX6
ENOB
HSPB1
PGM1
PGAM2
TPIS
FLNC
PEBP1
ACTS
TRFE
IGHG4
ALDOA
PROF1
ALBU
G3P
AT2A1
8
4
0
−4
−8
Synthesis difference (Log2)
−2
−1
0
1
2
Abundance change (Log2)

# Figure 10


A
Atrogin-1 mRNA (Arbitrary Units)
0.0
0.5
1.0
1.5
Pre
Post
a*
HFLC
REX
B
MuRF-1 mRNA (Arbitrary Units)
0.0
0.5
1.0
1.5
2.0
Pre
Post
*
HFLC
REX
C
Myostatin mRNA (Arbitrary Units)
0.0
0.5
1.0
1.5
2.0
Pre
Post
a*
HFLC
REX
D
FAT/CD36 mRNA (Arbitrary Units)
0.0
0.5
1.0
1.5
2.0
HFLC
REX
Pre
Post
E
GPR56 mRNA (Arbitrary Units)
0.0
0.5
1.0
1.5
Pre
Post
HFLC
REX
F
PDK4 mRNA (Arbitrary Units)
0
1
2
3
4
Pre
Post
HFLC
REX
G
PGC-1α mRNA (Arbitrary Units)
0.0
0.5
1.0
1.5
Pre
Post
a
a
HFLC
REX
H
PPARδ mRNA (Arbitrary Units)
0.0
0.5
1.0
1.5
HFLC
REX
a*
Pre
Post
I
IL-6 mRNA (Arbitrary Units)
0.0
0.5
1.0
1.5
2.0
2.5
Pre
Post
HFLC
REX

# Figure 11


A
p-4EBP1Thr37/46 (Arbitrary Units)
150
100
50
0
HFLC
REX
Pre
Post
B
4E-BP1 Total (Arbitrary Units)
150
100
50
0
HFLC
REX
Pre
Post
C
p-AMPKα Thr172 (Arbitrary Units)
80
60
40
20
0
HFLC
REX
Pre
Post
D
AMPKα Total (Arbitrary Units)
150
100
50
0
HFLC
REX
Pre
Post
E
p-mTORSer2448 (Arbitrary Units)
40
30
20
10
0
HFLC
REX
Pre
Post
F
mTOR Total (Arbitrary Units)
150
100
50
0
*
HFLC
REX
Pre
Post
G
p-p70S6KThr389 (Arbitrary Units)
60
40
20
0
HFLC
REX
Pre
Post
H
p70S6K Total (Arbitrary Units)
150
100
50
0
HFLC
REX
Pre
Post