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Original Article

Hylonome

Whey protein plus bicarbonate supplement has little effects on structural atrophy and proteolysis marker immunopatterns in skeletal muscle disuse during 21 days of bed rest

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Abstract

Objectives: To investigate the effect of whey protein plus potassium bicarbonate supplement on disused skeletal muscle structure and proteolysis after bed rest (BR). **Methods:** Soleus (SOL) and vastus lateralis (VL) biopsies were sampled from ten (n=10) healthy male subjects (aged 31±6 years) who did BR once with and once without protein supplement as a dietary countermeasure (cross-over study design). The structural changes (myofibre size and type distribution) were analysed by histological sections, and muscle protein breakdown indirectly via the proteolysis markers, calpain 1 and 3, calpastatin, MuRF1 and 2, both in muscle homogenates and by immunohistochemistry. **Results:** BR caused size-changes in myofiber cross-sectional area (FCSA, SOL, p=0,004; VL, p=0.03), and myofiber slow-to-fast type transition with increased hybrids (SOL, p=0.043; VL, p=0.037) however with campaign differences in SOL (p<0.033). No significant effect of BR and supplement was found by any of the key proteolysis markers. **Conclusions:** Campaign differences in structural muscle adaptation may be an issue in cross-over design BR studies. The whey protein plus potassium bicarbonate supplement did not attenuate atrophy and fibre type transition during medium term bed rest. Alkaline whey protein supplements may however be beneficial as adjuncts to exercise countermeasures in disuse.

Keywords: Bed Rest, Whey Protein, Disuse, Muscle Atrophy, Proteolysis Biomarkers

A loss of skeletal muscle mass occurs in various catabolic states such as cachexia, sarcopenia, and inactivity[1]. The principal change in muscle mass is related to altered muscle protein turn-over rates (imbalance between protein synthesis and degradation) for example shown following various periods of inactivity or unloading[2-5]. Which of the two key mechanisms of protein turnover, muscle protein synthesis (MPS) or muscle protein breakdown (MPB) might be more critical in disuse protein wasting is still not known[6-8].

Myofibrils comprise nearly 80% of skeletal muscle mass[9] and it is therefore no surprise that atrophy is largely due to protein wasting. The ubiquitin-proteasome pathway (UPP) and the calcium-activated calpain system are two key players in MPB. The UPP accounts for global proteolysis in normal and diseased muscle[10] while activated calpains and their endogenous inhibitor calpastatin, are involved in the proteolysis of functionally relevant structural proteins such as the cytoskeletal anchorage complexes[11] and myofibrillar proteins[12]. Intact myofibrils in a sarcomere are apparently "preprocessed" by the calpain system, which seems to be critical for providing access to the proteasome-mediated MPB[13]. The proteasome-dependent proteolysis is activated or inactivated during skeletal muscle

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overload or disuse, respectively[14]. Elevated ubiquitinated muscle proteins[15] and E3 ligase muscle RING-finger protein-1 (MuRF1) mRNA were shown to be proxies for increased protein breakdown following bed rest[16] or spaceflight[17]. Reliable serological muscle loss biomarkers are still not available to study dynamic muscle protein balance, for example, in human disuse[18]. Proteolysis markers thus still provide an indirect indication of MPB in disused human skeletal muscle.

High protein intake and essential amino acid supplementation are generally considered safe and helpful tools to induce anabolic cell signaling pathways to stimulate protein synthesis[19] in cachexia and sarcopenia[20] but also showed anti-catabolic effects on disused skeletal muscle following bed rest[21-24]. More recently, supplementation of whey protein as nutrient has been applied as an anti-catabolic regimen to overcome protein deficits or protein wasting in humans[25,26]. Rapidly digestible and leucine rich whey protein supplements were shown to enhance protein synthesis in human skeletal muscle[27-28]. Nonetheless, a disadvantage of a high leucine protein intake is the generation of acidic byproducts, i.e., sulphuric acid from sulphur containing amino acids in whey proteins[29]. As a consequence, whey supplementation potentially increases the renal acid load, and thereby leads to low-grade metabolic acidosis[30] that, in turn, increases MPB[31] that is a catabolic signal associated with muscle wasting[32]. The anabolic potential of a whey protein supplementation may thus be optimized by neutralizing this acidogenic effect by alkaline salts. In support of this it has been found that supplementation with whey protein and potassium bicarbonate (K-base, $KHCO_3$) maintained lean tissue mass in older humans[33]. However it is presently unknown whether such a combination has a therapeutic potential to attenuate atrophy and alleviate muscle protein wasting in disuse during bed rest.

Here, we report the effect of 21 days medium-term 6° head down-tilt bed rest (MTBR study) on structural, immunohistochemical and biochemical properties of disused skeletal muscle. This was investigated in a randomized cross-over controlled head-down tilt bed rest (BR) study with ten (n=10) healthy men[34].

Our main hypothesis was that whey protein plus $KHCO_3$ supplementation (i) helps to maintain structural myofiber properties (size and type distribution), and (ii) attenuate muscle protein breakdown detectable via skeletal muscle specific indirect proteolysis markers[35-37] during BR.

Materials and methods

Bed rest study

The 21 days 6° head-down-tilt (HDT) medium-term bed rest (MTBR/MEP-study) took place at the German Aerospace Center (DLR) in Cologne, Germany. The first campaign was performed in September and October 2011, and the second campaign in February and March 2012. The study was sponsored by the European Space Agency (ESA) and performed in accordance with the ESA bed rest standardization plan. The study was a controlled and randomized classical cross-over design. Energy- and nutrient intake were recorded, nutrition was controlled and standardised, and the intake of whey protein isolate+$KHCO_3$ prescribed as reported elsewhere[34]. Ten (n=10) voluntary healthy men (aged 23 to 43 years) were recruited and selected after a strict psychological, physiological and medical screening. Anthropometric data of the MTBR study are given in Table 1 and can also be obtained elsewhere (see www.clinical.trials.gov, Identifier: NCT01655979). Subjects were assigned to two groups and studied twice in two follow-up campaigns, once receiving a combination of whey protein plus $KHCO_3$ (K-base, bicarbonate) diet, and once receiving a standardized isocaloric diet as countermeasure[34]. For the 1st campaign, five of the participants (n=5) were randomly assigned to a bed rest-only control (CTR), and the other five participants (n=5) were randomly assigned to a bed rest intervention (NUTR) group. For the 2nd campaign (starting after a 12 weeks wash-out phase after the 1st campaign), the groups were assigned the other way around. In the 2nd campaign, biopsies were sampled except from two subjects (one had medical problems unrelated to the study and one withdrew from the study resulting in ten biopsied participants (1st campaign) and eight biopsied participants (2nd campaign)[34]. Peer review and ethical approval of the study were given by the Ethical Board of the Ärztekammer Nordrhein, Duesseldorf, Germany (**Me**dium-term whey **p**rotein [MEP] - bed rest study, #2010426 from 13/05/2011). The study adhered to the Declaration of Helsinki. Participants provided informed consent after study information briefings and were allowed to withdraw from the study at any moment.

Subjects (n=10)	1st campaign	2nd campaign*
Age (years)	31.6 ± 5	31.5 ± 5
Height (m)	1.80 ± 0.06	1.80 ± 0.03
Weight (kg)	76.1 ± 8	70.0 ± 6
BMI	23.43 ± 1	24.02 ± 2

*Cross-over design: 1st campaign (n=10); *2nd campaign (n=8) drop-out of one subject; BMI: Body Mass Index [kg · m-2]; for more details see Buehlmeier J et al.,[34].*

Table 1. Anthropometric data of subjects of the MTBR study (mean +/- S.D.).

Nutritional intervention

Nutritional intervention was a combination of whey protein (WP) plus bicarbonate (WP + BC-only, no exercise) supplementing the daily food intake[34]. The whey protein isocalorically replaced fat and carbohydrates. For the WP+BC group, named NUTR group throughout this study, supplementation of WP (0.6g · g^{-1} · body $weight^{-1}$ · day-1) resulted in total protein intake of 1.8 g · kg^{-1} · body $mass^{-1}$. WP was supplied by Diaprotein® (Dr. Steudle Inc.). Protein powder was dissolved in milk products. In addition to WP, the NUTR group also re-

ceived bicarbonate (BC, 6 x 15 mmol) in six portions within meals and snacks to achieve daily supplementation of 90 mmol $KHCO_3 \cdot day^{-1}$ (Krueger GmbH). The CTR-group received a standardized isocaloric diet to meet the basic nutrient recommendations. Further details on the diet that adhered to the standardisation plan of the European Space Agency (ESA), for example, subject-matched menus, matching energy intake to expenditure as calculated by PRODI® 5.6 software, and vitamin intake (recommended daily allowances), in particular Vitamin D supplementation (1.000 IE) due to missing UVB light in the bed rest facility, are described elsewhere[34]. Intake of caffeine, methylxanthine and alcohol was prohibited during the study.

Muscle biopsy

Muscle biopsies were sampled two days before the start of the bed rest period (preBR, BDC-2) and shortly before reambulation (postBR, HDT19) from the lateral aspect of the quadriceps femoris muscle (vastus lateralis [VL], phasic, mixed fast/slow type), and from the deep calf soleus muscle (SOL, postural, mostly slow-type) of the right leg. A Rongeur forceps (Zepf Medizintechnik) was used for skeletal muscle tissue sampling through an approx. 1 cm skin/fascia incision following local anesthesia (2 ml of 1% Lidocain). In total, seventy-two (n=72) individual biopsies were collected from either muscles from the 1st campaign subjects (n=10) or from the 2nd campaign subjects (n=8). The samples were subdivided into one histology piece (i.e., longitudinally fibre orientation assessed by optical stereoscopy) and several other tissue pieces (approx. 20 mg each) for biochemical and molecular analysis. The histology piece was embedded with O.C.T. compound (Scigen® Gardena) for better longitudinal orientation. All samples were immediately frozen in liquid nitrogen, while vigorously shaking, and stored at -80°C until further use.

Immunohistochemistry (IHC)

Serial 8 µm cross sections were cut in a cryotome at minus 20°C (CM 1860, LEICA Microsystems). The sections were mounted on coded glass slides (SuperFrost® Plus, 631-0108, VWR International) and incubated with one, two or three different primary antibodies. Primary antibodies used were anti-myosin-heavy chain (M8421 recognizes slow MyHC I, clone My32, M4276 recognizes fast IIa, IIb or IIx, Sigma), and mono- or polyclonal antibodies for the subsarcolemmal protein dystrophin (mono #107416, Novocastra, or poly SC-15376, Santa Cruz Inc.) according to previously published protocols[35]. The location of primary antigen binding was detected by fluorescent dye-conjugated secondary antibodies using red-fluorescent (Alexa Fluor 555 labelled goat-anti-mouse, A21421, and polyclonal goat-anti-rabbit, A21429, Invitrogen), -green fluorescent (Alexa Fluor 488 labelled mouse monoclonal, A11025, and polyclonal goat-anti-rabbit, A11034, Molecular Probes), or blue fluorescent (Alexa Fluor 635, labelled, A31575, goat anti-mouse, Molecular Probes). Fibre cross-sectional area (FCSA) and myofibre type distribution (type I vs. type II) were calculated/determined in groups of transversely cut myofibre profiles outlined by dystrophin-immunopositive sarcolemma membrane within the region-of-interest (ROI) using LEICA built-in software. FCSA is given in square microns (μm^2). Fibre type distribution was assessed by individually counting myofibres immunoreactive for MyHC type I, MyHC type IIa, IIx or hybrid fibres (MyHC I/MyHC II co-immunoreactivity in myofibre profiles). All determinations were done in triplicate according to statistical protocols as previously published[36]. A minimum of 30 myofibres from each subject, muscle and BR condition were screened to determine the proportions and CSA of each fibre type. Routine epifluorescence microscopy (ZEISS Axioplan) and/or high-resolution three channel (HeNe [543 and 633 nm] and Argon [453 nm]) multilaser confocal analysis (TCS SP-2, LEICA) were used for subject-matched semi-quantitative image analysis of immunohistochemistry. A pair-wise analysis of post vs. pre bed rest cryosections from identical subjects immunostained in the same incubation protocol was performed to avoid any bias between immunohistochemical protocols and to balance inter-subject variability[39].

Myosin heavy chain composition

The myosin heavy chain (MyHC) composition was determined as previously reported[37]. The separating and stacking gel contained 30% glycerol and 7% and 4% polyacrylamide, respectively. One 10 µm biopsy cryosection was dissolved in 100 µL sample buffer (Laemmli) of which 10 µL was loaded onto the gel. The gel run for 27 h at 15°C and bands were visualized with the Silver Stain plus kit (Biorad), and identified (type I, IIx and IIa) based on their migration distance. Quantity One™ (Biorad) was used to determine the relative proportion of each of the MyHC isoforms, using the lane density profile.

Protein assays

Small amounts of muscle samples (approx. 3-5 mg wet mass) from each subject and muscle were lysed 1:10 with RIPA-buffer (50 mM TRIS, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM $MgCl_2$, plus Complete Mini with pepstatin and sodium fluorate, and benzoase, pH 7.3-7.4). Total protein concentration in homogenates was determined in supernatant by the colorimetric BCA-Protein Assay (Pierce Inc.) and presented as $mg \cdot ml^{-1} \cdot$ g wet $mass^{-1}$ for each sample.

Protein biochemistry and Western Blotting (WB)

Muscle biopsy lysates ($1 mg \cdot ml^{-1}$) from pre and post subject-matched samples were loaded on parallel lanes at identical protein titers ($3 \mu g \cdot \mu L^{-1}$) on polyacrylamide gels and electrophoresed and blotted as previously described[36]. Alpha tubulin served as housekeeping protein. Briefly, MuRF1 and MuRF2 mono/polyclonal antibodies (Abcam ab57865 and ab4125), and a second poly MuRF-2 antibody (kindly gift of S. Labeit, Mannheim, Germany) were used as previously described[38] for WB with human tissue. Anti-calpain 1 (poly-rabbit, #28258), -calpain 2 (poly-rabbit, ab39165), and -calpastatin (poly-chicken,

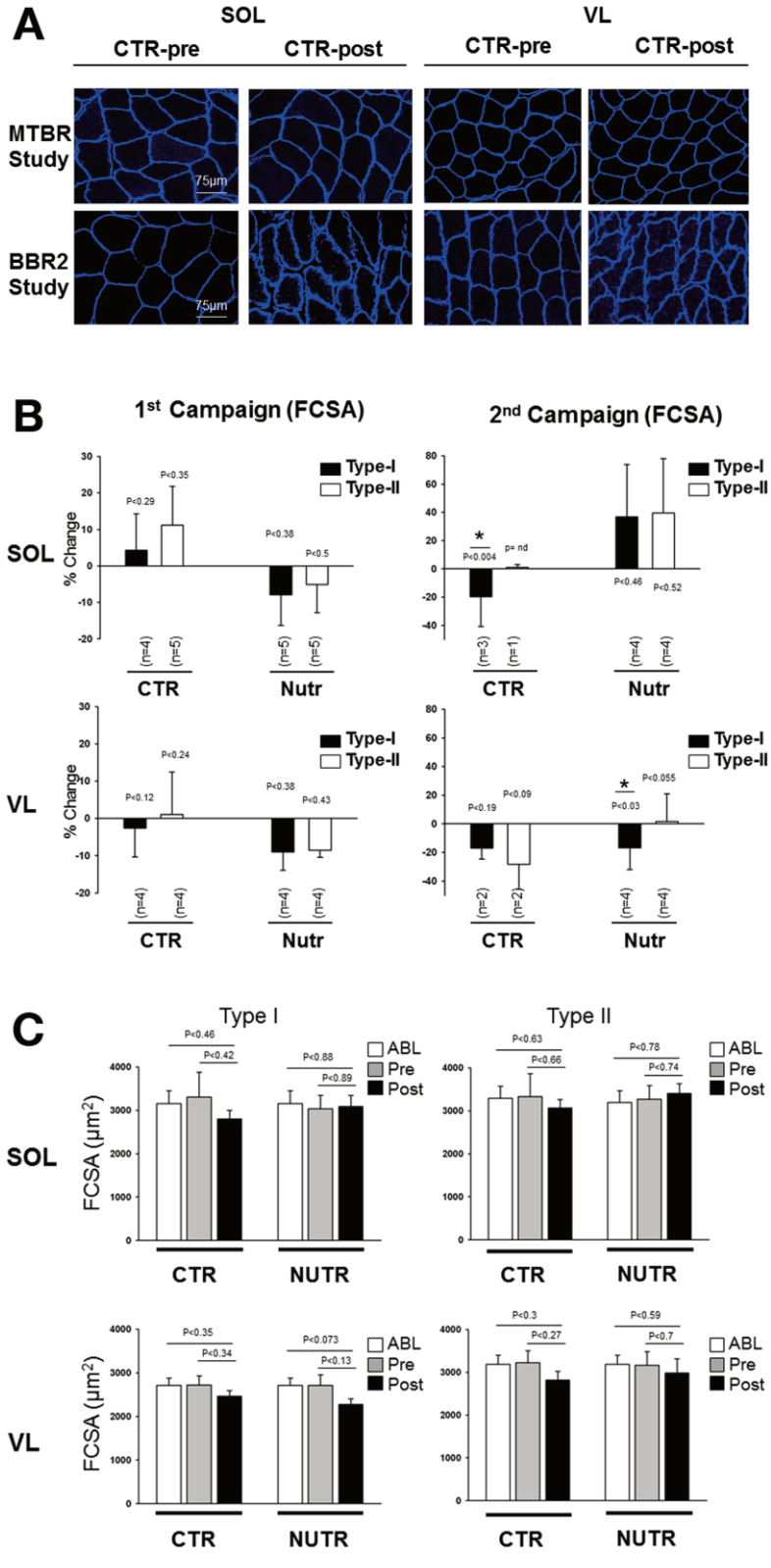

Figure 1. Dystrophin immunohistochemistry in bed rest muscle biopsies and fiber cross-sectional area (FCSA) determination in MTBR study samples. **A.** Soleus and vastus lateralis cross-sections immunostained for dystrophin from 21-days mid-term (MTBR) study (upper panel), and from the 2nd Berlin BedRest (BBR-2) study (lower panel) at bed rest start (CTR-pre) and end (CTR-post). Bars=75 µm (for all). **B.** Bar graphs showing campaign-wise (1st and 2nd campaign) quantitative determination (% change FCSA, µm² from baseline) in SOL and VL myofiber types in the MTBR study groups (CTR vs NUTR). **C.** MTBR study FCSA data (pooled raw data) in both muscles, fiber types (type I and II) vs. study groups. SOL=soleus; VL=vastus lateralis, ABL=average baseline; nd=not determined.

Soleus (SOL)				Vastus lateralis (VL)			
CTR (n=8)		**NUTR** (n=10)		**CTR** (n=8)		**NUTR** (n=10)	
Pre	Post	Pre	Post	Pre	Post	Pre	Post
574.6	557.9	544.7	564.2	636.6	655.9	571.8	589.1
±24	±23	±17	±19	±20	±25	±13	±12
P<0.627#		P<0.465#		P<0.567#		P<0.358#	
P<0.996*		P<0.800*		P<0.05*		P<0.583*	

Pre/Post values represent means (mg · ml^{-1} · g wet mass^{-1}) ± SEM.
*Significance at P<0.05; #ANOVA (within group) post vs. pre; *ANOVA (between groups) vs. average baseline (pre) of SOL and VL.*

Table 2. Relative protein concentration (biopsy).

ab16423) were from Abcam Inc. We used secondary alkaline-phosphatase conjugated antibodies (swine-anti-rabbit, #D0306, DACO; goat-anti-rabbit, Sc-2928, Santa Cruz Inc.). Reflexive densities (RD) were measured in WB by densitometry scanning (G-800, Quantity One™ Protein Analysis software, Biorad Inc.) normalized to tubulin content (anti-alpha-tubulin, monoclonal, #T6199, Sigma). Mean RD values (arbitrary units) from individual CTR or NUTR groups were tested against average baseline (ABL) values calculated from the pooled Pre values of either groups (CTR-Pre plus NUTR-Pre).

Statistics

Data are presented as mean ± standard deviation (S.D.), or standard error of means (S.E.M.). The baseline data for the first and second campaign were either analysed subject-matched (pre vs. post) or pooled (group-wise), and differences between baseline, 21 days bed rest (CTR) and 21 days bed rest + supplement (NUTR) were tested by paired Student´s t-test (Sigma Plot 9.0 or SPSS 19.0). A repeated measures ANOVA with muscle as within factor (SOL vs. VL) and between factor condition (ABL, CTR, NUTR) was used. Differences were considered significant at $p<0.05$.

Results

Sarcolemma microstructure in myofibres

Immunohistochemical appearance of dystrophin identified typically "ring-like" immunopatterns in most of the cross-sectioned and subject-matched CTR-post vs CTR-pre soleus and vastus lateralis (VL) biopsy reflecting an intact outer membrane with regular subsarcolemma microstructures present in less atrophic MTBR myofibres after 3 weeks in bed rest (Figure 1A, upper panel). For comparison, irregular dystrophin immunopatterns are clearly evident in highly atrophic soleus muscle fibres from a 8 weeks long-term bed rest (2nd Berlin BedRest, BBR2)[35] study suggestive for structural perturbations in highly atrophic myofibres that however were absent from the 3 weeks MTBR study samples (Figure 1A, lower panel).

Myofibre phenotype and size

We next documented changes in the myofibre cross-sectional area (FCSA) in triple-immunostained transversely cut myofibre profiles by individual quantitative analysis in SOL and VL for each subject of the MTBR study (Figure 1B). We found variable results showing within-group variability in all muscle samples and type I or II myofibres from the CTR group of 1st campaign participants (n=4, n=5) and significant changes (reduced FCSA) only for myofibres I in SOL of 2nd campaign participants (n=3, n=4) (Figure 1B). In VL muscle, there was a change in FCSA of myofibre type I from the NUTR group only from 2nd campaign participants (n=4) not seen in the NUTR group from the 1st campaign (n=4). Such changes found between campaigns were no longer observed after pooled data analysis (Figure 1C).

We then analysed the myofibre type distribution in SOL and VL biopsies from the MTBR study using MyHC immunohistochemistry for the two major MyHC subtypes, slow (type I) and fast (type IIa, IIx). Hybrid myofibres (asterisks) were positive for both MyHC immunolabels (Figures 2A and 2B). In SOL of CTR group we found a trend for a myofibre I to II transition as reflected by the presence of only a few hybrid fibres (<2% vs normal baseline) in all subjects at the end of MTBR (Figure 2C). In SOL and VL of the NUTR group, however, we found an increase in hybrid fibre formation (>40% to 80% vs. baseline) mainly in the 2nd campaign subjects reflecting ongoing myofibre remodeling (Figure 2C). However, the pooled data analysis given as pie graphs (Figure 2D) did not reveal changes in hybrid fibre quantities between study groups observed after campaign-wise analysis.

Muscle total protein extracts

In general, global screening of relative muscle protein content from the MTBR biopsy samples revealed no significant changes in the total amount of RIPA-buffer soluble proteins between pre and post samples, except those found in VL from CTR group if compared to average baseline suggesting the homogenous quality of the biopsy samples (Table 2).

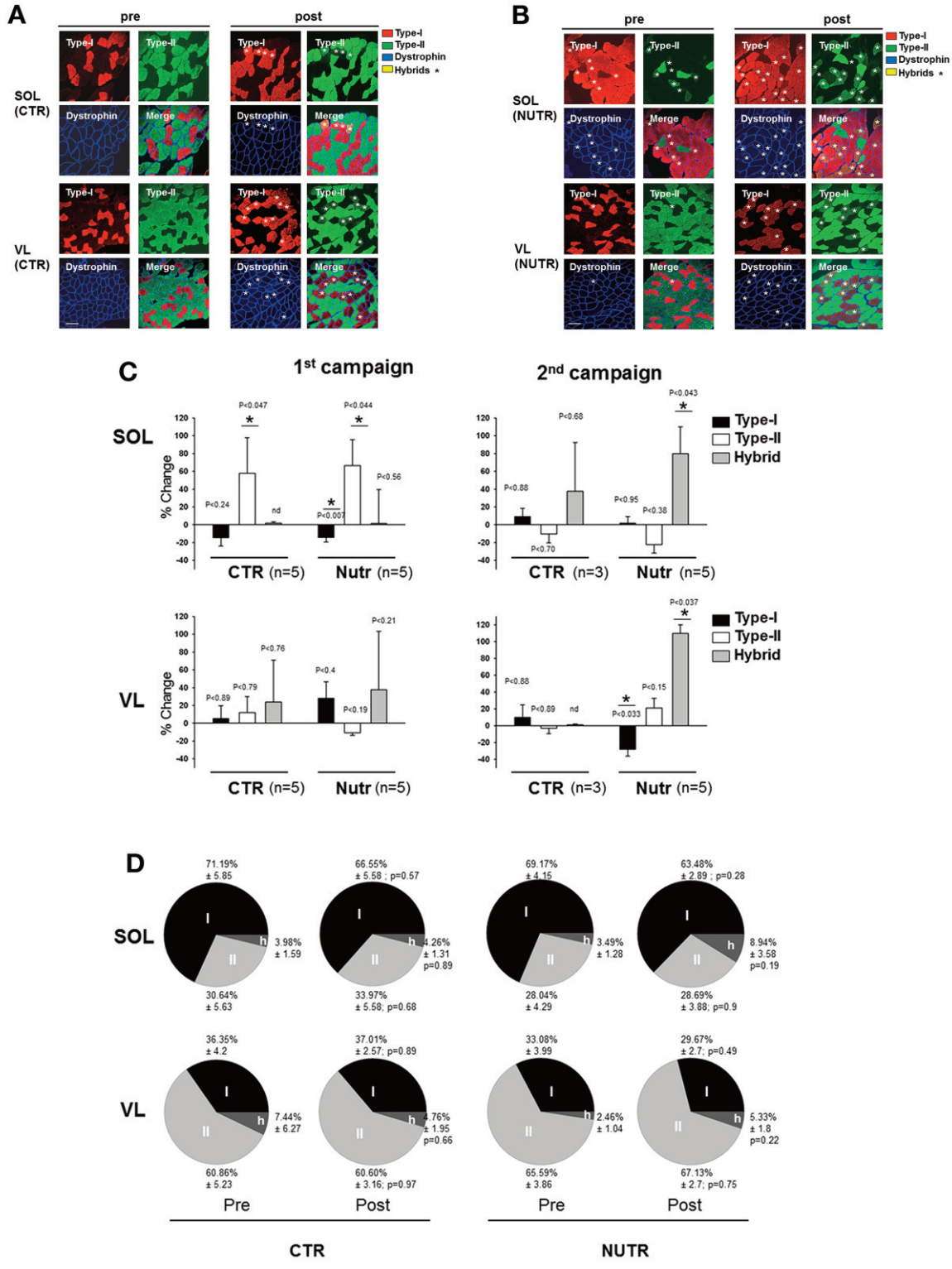

Figure 2. Myosin-heavy-chain (MyHC) immunohistochemistry and quantitative myofibre type distribution analysis of MTBR study samples. **A.** Control (CTR) group: Soleus (SOL, upper two panels) and Vastus lateralis (VL, lower two panels). **B.** Nutrition (NUTR) group: SOL and VL (upper and lower two panels). Triple immunostaining of cross-sections (slow MyHC I [red] / fast MyHC II [green] / dystrophin [blue]) shown as individual (red-green-blue), and merged (red-green-blue-yellow) confocal images. Asterisks (*) denote hybrid fibres (slow/fast MyHC co-immunolabel). Bar=200µm (left lower panel, for all). **C.** Quantification (% change vs pre) of 1st and 2nd campaign myofiber type distribution based on MyHC I, MyHC IIa/IIx, and hybrid fiber immunopatterns (cf. **A, B**). **D.** Quantification (pie chart, pooled study data) of SOL (upper panel) and VL (lower panel) muscle type I, type II or hybrid (h) myofibre distribution (given as %) determined by group-wise (CTR vs NUTR) differential MyHC I/II/h immunopositive myofibre counts in histological cross-sections. P-values, see graph; nd=not determined.

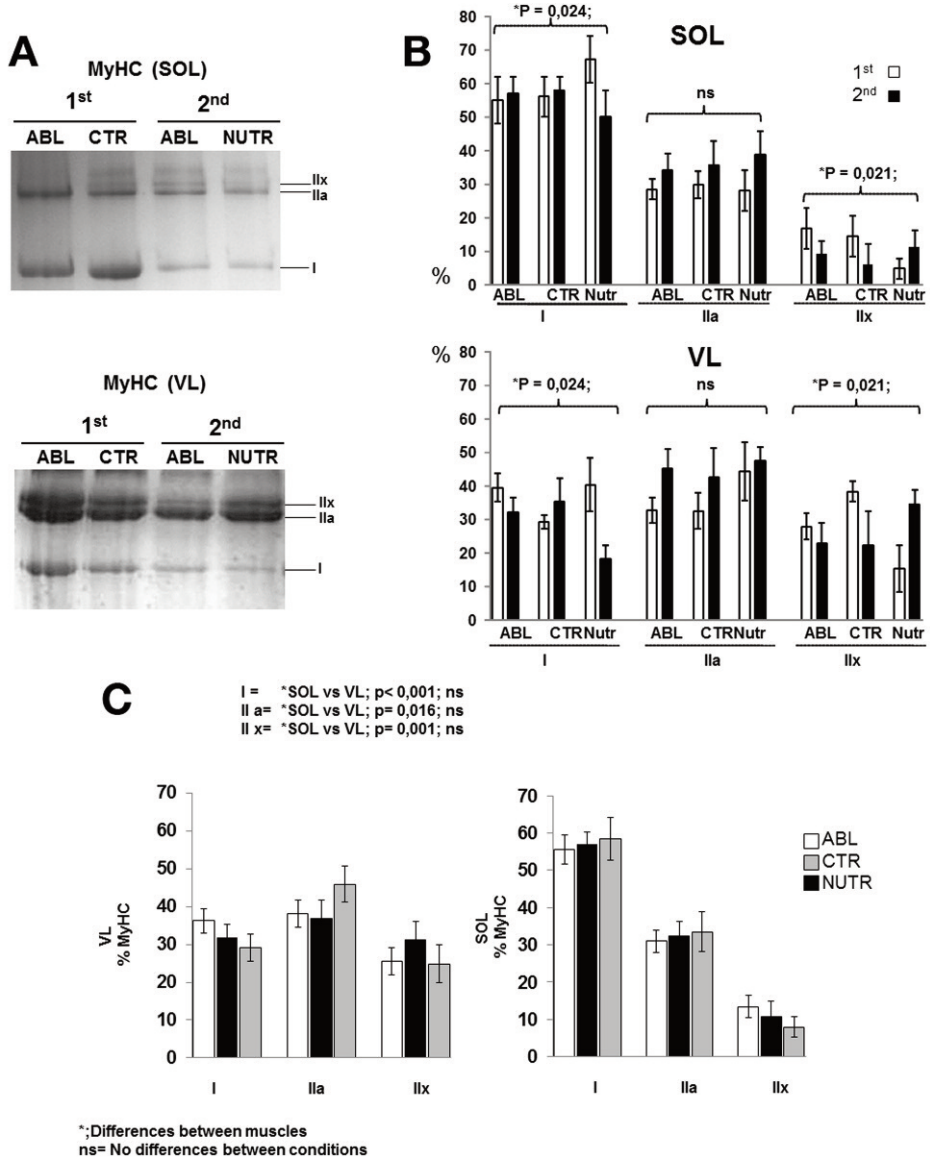

Figure 3. Relative myosin heavy chain (MyHC I, IIa, IIx) isoform composition in MTBR study samples. **A.** Silver-stained SDS-PAGE of 1st and 2nd campaign samples (overview). **B.** Bar graphs showing campaign-wise % differences (open vs. black columns) in MyHC I ($p<0.024$) and MyHC IIx ($p<0.021$) composition between muscle types (VL vs. SOL). **C.** Bar graphs (pooled study data) of muscle-specific MyHC isoform composition (VL vs. SOL) in CTR (grey bars) vs. NUTR group (black bars) and average baseline (open bars). ABL=average baseline.

Biochemical analysis of MyHC protein isoforms

As expected the proportion of MyHC I proteins determined by SDS-PAGE were larger in the soleus than the vastus lateralis ($p<0.001$) vs. baseline (Figure 3). The relative proportion of MyHC 1 ($p<0.024$) and MyHC IIx ($p<0.021$) was variably changed (higher or lower vs baseline) in both muscles particularly in the 2nd campaign NUTR group subjects (Figure 3B). Pooled data analysis (Figure 3C) showed changes in both MyHC IIa ($p<0.028$) and MyHC IIx ($p<0.004$), but not in MyHC I ($p<0.001$). Though differences in muscle were obvious (as reflection of the known myofibre I to II transition) a statistical difference between the two bed rest groups (CTR or NUTR) was not found by pooled group analysis with respect to MyHC isoform composition in soleus or vastus lateralis (Figure 3C).

Proteasome-based protein breakdown markers (MuRF1 and MuRF2)

No changes in MuRF1 (not shown) or MuRF2 immunohistochemical expression levels were present in the myofibres from the 21 day MTBR study groups (Figure 4A, upper panel). For comparison (Figure 4A. lower panel), a strong cytosolic MuRF2 immunohistochemical detection pattern is found in at-

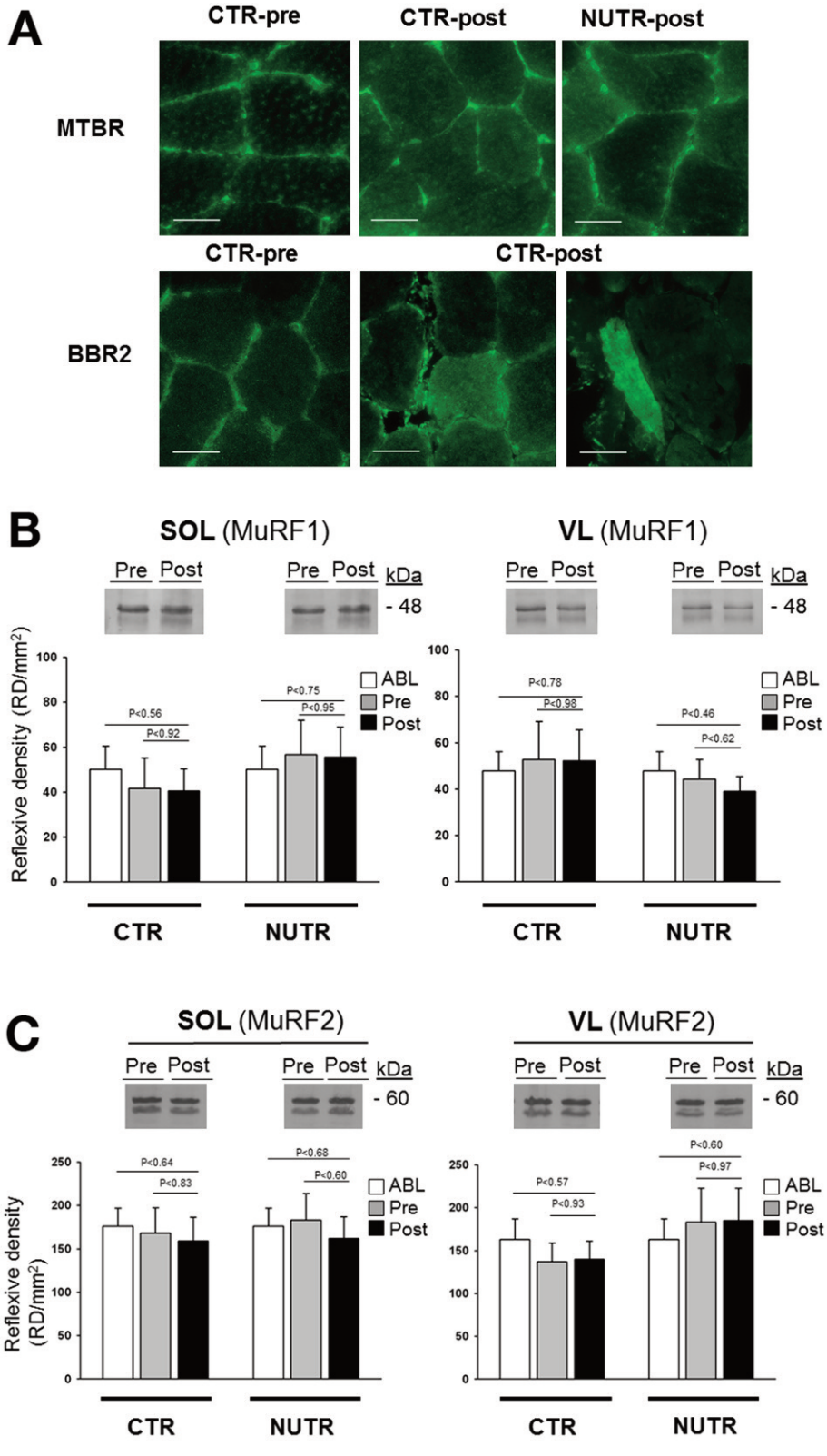

Figure 4. MuRF-related proteolysis markers in human soleus biopsy from a medium-term (21d MTBR) vs. long-term bed rest study (60d BBR-2) at start (pre) and end (post). **A.** MuRF2 immunofluorescence, upper panel (CTR-pre vs CTR-post and NUTR-post), lower panel (BBR2 study, CTR-pre vs. post, two examples). CTR = control group, NUTR=nutrition group. Bars=75 μm. **B and C.** MTBR study: Quantitative subject-matched (Pre vs. Post sample lanes, 48 kD) immunoblot analysis for MuRF1 (B), and MuRF2 (C) in pre vs. post SOL and VL (grey and black columns) vs. average baseline (open bars). SOL=soleus, VL=vastus lateralis, ABL=average baseline; RD=reflexive density per square milimeters (RD · mm^{-1}). P-values, see graph.

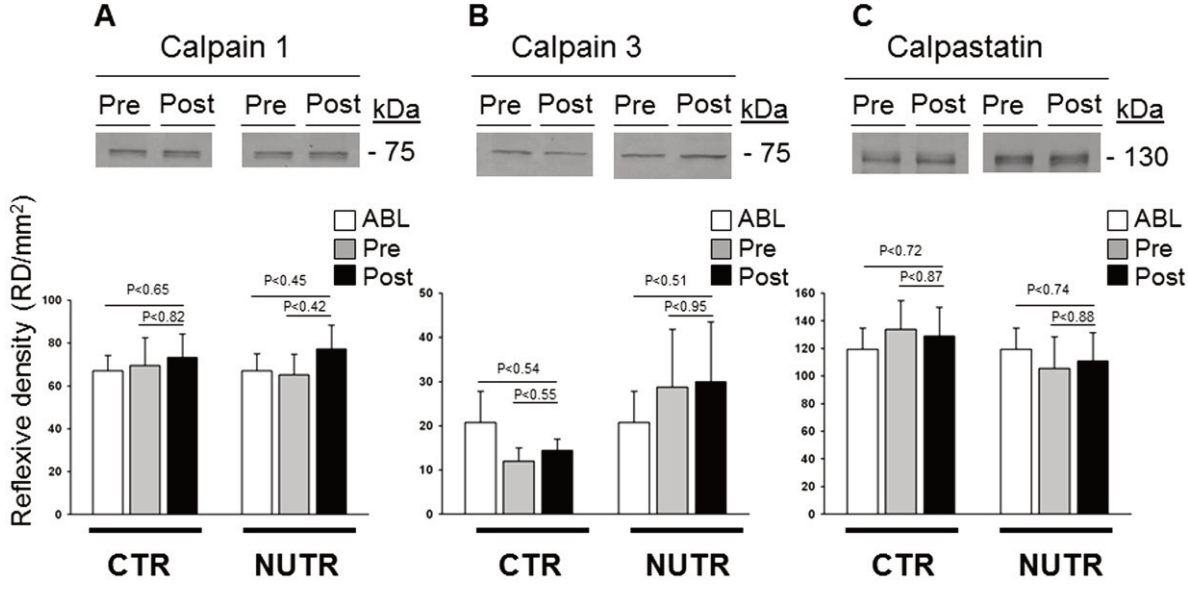

Figure 5. Calpain-related proteolysis markers in MTBR study biopsies (SOL). Upper panel: representative subject-matched Pre vs. Post sample lanes of the immunoblot analysis for calpain 1 (**A**), calpain 3 (**B**), and calpastatin inhibitor (**C**). Lower panel: bar graphs showing quantitative immunoblot analysis between pre and post study groups (grey and black columns) compared to average baseline (ABL, open bars), RD=reflexive density per square milimeters (RD · mm^2). P-values, see graph.

rophic SOL myofibres of a 60 day BBR2 study biopsy (Figure 4A, lower panel). These immunohistochemical results were largely supported by the biochemical MuRF1 and MuRF2 immunoblot analysis (Figure 4B and C) by using the same anti-MuRF1 and anti-MuRF2 antibodies that both showed little proteolysis marker changes in either muscle (SOL, VL) or bed rest conditions.

Calpain-based protein breakdown markers (Calpain 1, Calpain 3, and Calpastatin)

Immunoblot analysis of subject-matched MTBR soleus lysates were performed using calcium-dependent cystein protease markers, anti-calpain 1 (Figure 5A), -calpain 3 (Figure 5B), and endogenous calpain-specific inhibitor protein marker anti-calpastatin (Figure 5C). Quantitative analysis did not show significant changes between groups (0.95<p>0.42) compared to average baseline (ABL) values in each CTR or NUTR group (Figure 5A-C).

Discussion

Bed rest is an established experimental model to simulate the physiological effects of disuse on the human organism[39] and represents a key model for studying muscle structural and physiological adaptation to deconditioning that may occur in various clinical settings including critical care[40], but also following microgravity exposure in Space[41-43]. Apart from often strenuous and high intensity exercise prescriptions that may not always be well tolerated in muscle during periods of disuse, alternative countermeasure protocols including nutritional supplementation could be a reasonable and feasible approach to a reduction of muscle wasting in prolonged inactivity in bed rest, critical illness or spaceflight[19]. The present MTBR study was designed to study the effects of a promising new combination of whey protein plus bicarbonate as nutritional countermeasure to impede disuse-induced skeletal muscle atrophy following 3 weeks of bed rest.

In the present study, we found no significant changes in muscle protein breakdown levels in bed rest as measured indirectly by calpain 1, calpain 3, calpastatin, MuRF1 or MuRF2 antigen markers either used for immunohistochemistry or in immunoblots. Recent findings suggested that calpains activated by PGC1α[44] for example via elevated cytosolic calcium in disused mouse soleus[45] may act "upstream" of the UPP by activating proteasome-dependent proteolysis and inhibiting the Akt/mTOR signaling pathway of protein synthesis[46-47]. The lack in calpain/MuRF-related proteolysis marker changes found in the present MTBR study in humans coincides with the proposed idea that MPB may not always contribute substantially to human skeletal muscle in disuse[39]. Perhaps, alternative pathways of muscle protein degradation (e.g., via lysosomal cathepsin proteases, SUMOylation, glucuronidation, or autophagy) may equally be at work following disuse to be further investigated in forthcoming bed rest studies.

Based on the present outcome and on recent reports either variable protein turn-over, subject variability or experimental design of studies on healthy humans are even more critical determinants[48] particularly under various periods of disuse. Protein turn-over appears to vary during the time course of

disuse[49] or may also be affected by the timing of protein administration during or after a period of disuse[50-51]. Short-term muscle disuse (1 to 7 days) resulted in more ubiquitinated muscle proteins[52] and early acute atrophy effects were frequently reported only at mRNA and molecular signaling pathway level by altered MuRF1/MAFbx transcript levels following a few days of human muscle disuse after cast immobilization[53]. The rise in protein breakdown may also imbalance protein synthesis that may both converge in ongoing muscle atrophy thus mediating the rate of muscle atrophy in short (<10 days) or medium-term duration (>10 days) disuse periods[54]. Changes in expression levels of MuRF-mRNA were indeed recently found in cast immobilized human skeletal muscle after short disuse (5 days)[52] and in knee braced disuse in young or old healthy men (4 days)[51]. These changes were also associated with impaired single fibre contractile function[53], data suggesting initial molecular atrophy changes to occur prior to onset of structural atrophy (reduced myofibre size, myofibrillar protein wasting) of disused human skeletal muscle. It is suggested that ubiquitin protein conjugates and related proteolysis markers (MuRFs, atrogins) acutely upregulated within the first days of immobilization and may return back to steady state levels within two weeks[39,51]. If we consider a 20 days estimated half-life time span of human muscle proteins determined from isolated fibre bundles[53] one *in vivo* "completed" muscle protein turnover cycle would have been terminated at least in the range of about two to three weeks depending on normal activity levels or disuse in bed rest. Even more, both hypocaloric and hypercaloric nutrition may affect MPB as previously shown in bed rest[55-56] suggesting isocaloric (normocaloric) nutritional intervention (this study) as a standardized requirement for studying protein balance in human nutritional intervention bed rest studies. Further time course studies are necessary in order to unequivocally address the still puzzling human muscle protein turnover and wasting and its tightly linked moderation by specific protein supplementation, for example, testing them in various durations of bed rest.

Physical inactivity alone may account for a recently termed "metabolic inflexibility" including insulin resistance observed after muscle inactivity such as in bed rest[57]. A disturbed regular amino acid/protein uptake during feeding known as "anabolic resistance" suggested adaptive depression rather than increases of muscle proteolysis as a critical mechanism to impaired homeostasis in regular muscle mass in disuse[58]. It is also suggest that yet unknown signaling pathways inherent to muscle sensing a "muscle-full state" might result in anabolic resistance to muscle protein synthesis[59].

At first sight, we may think that the obvious lack of any structural myofiber atrophy (size or type changes) and little if any signs of protein degradation (via indirect immunohistochemical and biochemical protein breakdown markers) would be the result of BR and/or the specified alkaline protein supplement administered during BR. Unlike previously shown in 60 or 90 days long-term BR[35-36], significant structural atrophy (reduced myofibre FCSA profiles demarcated by dystrophin marker) was not really observed in the present medium-term BR study with the post biopsy sampled at BR+19 (i.e., 19 days MTBR study). As part of a multiglycoprotein subsarcolemmal scaffold complex (DGC) dystrophin is considered a key functional protein necessary for mechanical linking of intramyocellular contractile forces via actin to extracellular matrix proteins such as laminins[60]. Intact dystrophin scaffold is thus crucial for membrane stability in normally active skeletal muscle and in force transduction[61]. We think that altered dystrophin immunopatterns seen in the myofibres after chronic disuse (e.g., 8 weeks, Berlin BedRest Study, BBR-2)[35] are histomorphological signatures of progressive structural atrophy (e.g., myofibrillar protein wasting) in shriveled myofibres possibly due to an "oversized" outer membrane envelopment with altered mechanical properties) that, however, was not seen after only 3 weeks of disuse in the present MTBR study.

A slow-to-fast myofibre type transition is a well-known phenomenon typically found after longer periods of human skeletal muscle disuse and also found in the present 21 days bed rest study. Nevertheless, even after 5 weeks of disuse many fibres were still observed in a transitory state (expressing both MyHC I and II proteins) compared to much earlier detectable MyHC mRNA and gene transcriptional changes found in the human vastus lateralis prior to MyHC protein expression with an apparent mismatch found in bed rest[62].

The present data analysis revealed some peculiar results related to myofibre phenotype distribution in skeletal muscle from both MTBR campaigns subjects who, however, revealed within-group variations. Increased hybrid fibre formation seen in the MTBR study likely reflects ongoing tissue remodeling with yet incompleted fibre transition (slow I-to-fast II) particularly in the SOL of the 2nd MTBR campaign subjects. In VL, however, the 1st campaign NUTR group showed a trend to delayed myofibre transition, but this effect was found significant only in the 2nd MTBR campaign NUTR group. A three months interim phase between the 1st and 2nd MTBR campaign is usually considered sufficient for the randomized cross-over design study[34]. Due to the present findings, however, a straightforward explanation for such differences might be that the interim phase of three month probably was too short to wash-out all campaign effects. In other words, the presently accepted notion of a simple "reset" of structural and functional or even molecular properties inherent to skeletal muscle tissue back to baseline levels, for example, in cross-over design BR study groups with their skeletal muscles challenged by immobilization and re-mobilization or vice versa should be critically assessed for future BR studies.

Gain or maintenance in muscle mass is known to be likely controlled by activity levels[63-64]. The aim of the study was to clarify whether or not a protein-based nutritional intervention on its own may be effective as a reliable anti-catabolic strategy to overcome disuse-induced atrophy in healthy subjects. Our results indicate that this was not the case. A recent study in older (50±8 years) exercised and young (21±8 years) untrained humans consuming protein supplements after aerobic exercise did not increase rates of mixed muscle protein synthesis over 6 weeks despite the MPS was higher than in the younger

sedentary group[65]. Another study with young untrained men showed that whey protein initiates protein translation in exercised skeletal muscle via the mTOR-dependent signaling pathway[66]. The absence of mechanical loading during the bed rest period and/or even a variable activity status of the participants before study start or even during the interim period between campaigns might account for the unexpected outcome of the MTBR nutrition-only study that showed little if any changes in the structural myofibre properties.

We conclude that alkaline whey protein supplementation showed only marginal changes at least on structural myofibre properties with possible delayed hybrid fibre transition in some but not all subjects throughout the bed rest periods that, in turn, is possibly also due to some unexpected campaign differences (carry-over effects) explored in the present study. The present study also revealed no major changes in key proteolysis markers in biopsy material from two different reference skeletal muscles suggesting little if any proteolysis to occur above constitutive protein turn-over in disused human skeletal muscle after three weeks of bed rest. Based on the present study outcome we suggest that future studies aimed at investigating anabolic compounds in disuse may require longer interim phases between campaigns, a strict control regarding habitual activity profiles, including subject monitoring also during the interim phase of a cross-over study design, in addition to monitoring ambulatory period activity levels[34]. Countermeasure trials using whey proteins to moderate disuse atrophy should be tested in longer duration bed rest disuse (>3 weeks) probably combined with mechanical muscle loading as additional anabolic trigger. The study findings may have wider implications for therapeutic potential studies of sustained protein wasting after periods of inactivity, cachexia or sarcopenia.

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