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DETERMINANTS OF INTRA-INDIVIDUAL VARIATION IN ADAPTABILITY TO RESISTANCE TRAINING OF DIFFERENT VOLUMES WITH SPECIAL REFERENCE TO SKELETAL MUSCLE PHENOTYPES

Determinants of intra-individual variation in adaptability to resistance training of different volumes with special reference to skeletal muscle phenotypes

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Abstract

The preface pretty much says it all.

Second paragraph of abstract starts here.

List of scientific papers

I. **Hammarström D**, Øfsteng S, Koll L, Hanestadhaugen M, Hollan I, Apró W, Blomstrand E, Rønnestad B, Ellefsen S Benefits of higher resistance-training volume are related to ribosome biogenesis. The *Journal of physiology.* 2020 Feb;598(3):543-565. doi: 10.1113/JP278455.

II. Khan Y, **Hammarström D**, Rønnestad B, Ellefsen S, Ahmad R Increased biological relevance of transcriptome analyses in human skeletal muscle using a model-specific pipeline. *BMC Bioinformatics.* 2020 Nov 30;21(1):548. doi: 10.1186/s12859-020-03866-y

III. **Hammarström D**, Øfsteng S, Jacobsen N, Flobergseter K, Rønnestad B, Ellefsen S Ribosome accumulation during early phase resistance training. *Manuscript*

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1. Introduction

Skeletal muscle health is essential for physical independence. In a lifespan perspective, measures of muscle mass and/or strength are inversely associated with mortality [1, 2, 3, 4, 5, 6, 7] and disability [8]. Besides adverse associations between of low muscle mass and strength and clinical conditions, muscle weakness also accounts for increased health care costs in patient populations [9, 10]. The intercept between muscle mass, muscle function and health status is interrelated with variables such as age and primary illness or injury [11]. This highlights that interventions designed to increase muscle mass and strength are likely to prevent adverse health outcomes across the lifespan. A higher level of muscle mass and functional capacity would counteract the effects of muscle loss due to illness, age or inactivity.

Although a large degree of the observed variations in lean mass and strength are attributed to genetic components [12, 13], environmental factors also contribute, leaving a window of opportunity to increase muscle mass and functional capacity. Among factors affecting muscle mass and functioning are nutrition and pharmacological agents. However, physical activity and specifically systematic resistance training of sufficient volume, intensity and frequency provides a stimulus that promote morphological and functional changes to the human neuromuscular system without adverse side-effects. Irrespective of age, resistance training generally leads to increased muscle mass and strength [14, 15] and is considered safe when performed in a well organized manner [15, 16].

Resistance training can be modulated indefinitely through combined variations of training variables such as frequency, intensity and volume [17, 18]. Well designed training prescriptions should incorporate information about the current state and goals of the trainee to maximize the potential outcome of the training program [17, 19, 18]. Training volume has received particular attention in the scientific community for many reasons. Evidence suggests that exercise volume affects selected molecular determinants of muscle hypertrophy in a dose-dependent manner [20, 21, 22]. Such effects are believed to facilitate long-term training effects as training programs with higher volume generally result in higher gains in muscle mass

and strength with little evidence of differences between age groups or participants with different training backgrounds [23, 24, 25].

A consequence of a more extensive training program is the increased time required to complete such a program. As time constraints has been reported as a limiting factor for engaging in physical activity [26] some merit can be given to arguments against guidlines suggesting higher volume in resistance training prescription [27, 19]. From an individual perspective, training prescription that balances time-requirement with efficacy presumably increases the likelihood of participation in physical activity [26]. From a more general perspective, increased knowledge about mechanisms governing responses to physical training could improve training prescription also for individuals and populations that experience attenuated benefit of resistance training [28]. The overreaching goal of the present thesis is to contribute to understanding individualized training loads. To this end, training volume was used to study the effects of variable training stimulus in within-participant models of exercise-training.

2. Background

2.1 Resistance-exercise prescription, a historical note and current challenges

Recommendations of systematic physical exercise with the purpose of improving health or physical performance has long been part of human culture, evident from records dating back to ancient Chinese, Indian and Greek civilizations [29]. Today's exercise-training prescription still bear traces of ideas from these eras, further developed during the renaissance and formalized in systems like German Turnen and Ling gymnastics during the nineteenth century [30]. German Turnen as a system of physical activities was established in a time when Germany developed from aristocracy to a unified nation. Turnen not only served as a system of preparing men to fight for the developing nation but also as a way of establishing a national identity. Ling gymnastics shared common origins with German Turnen and also served as a system of military preparation. However, Ling also established systems for medical, pedagogical and aesthetic gymnastics. Especially Ling's medical gymnastics was important for the development of modern exercise prescription as it was scientifically oriented, based on the physiological and medical understanding of that time [30]. The medical gymnastics of the nineteenth century is referenced in twentieth century texts on therapeutic exercise prescription [31].

With the introduction of "heavy resistance exercises" for development of muscle strength and mass after injury, DeLorme outlined a system on which modern resistance-exercise prescription is based [32]. DeLorme published his system short after the Second World War [32] during which he, as a newly graduated physician, had been working with war injury rehabilitation [33]. Inspired by practitioners of weight training [33], DeLorme specifically emphasized high-resistance, low-repetition exercises where progression was achieved with increased resistance [32] as opposed to previous recommendations of endurance-like exercise where progression was achieved through increased number of repetitions [31]. DeLorme originally used the term "heavy resistance exercises" in contrasts to low-resistance exercises [32],

but as this could be perceived as exercises performed only with heavy weights, the system was renamed *progressive resistance exercise* to better reflect the method [34][1] . Indeed, central to the system was the concept of repetition maximum [32]. Repetition maximum refers to the external resistance that can be overcome with a given number of repetitions. By adjusting external resistance to each individuals progression over the course of a training program, exercises are both individualized and progress is monitored [32]. DeLorme originally prescribed sessions of up to 100 repetitions performed in sets of 10 repetitions [32] but later revised this recommendation to three sets of 10 repetitions performed with increasing intensities [34].

Scientific inquiries into prescription of resistance training from the first part of the twentieth century concerned its therapeutic use [e.g. 32, 35] but was also evaluated in the context of improving strength and physical performance in healthy populations [e.g. 36, 37, 38]. Scientific contributions soon moved from questions regarding the effectiveness of resistance training *per se* to comparing outcomes from different modes of resistance training [39, 40, 41, 42, 43, 44]. A vocabulary for progressive resistance exercise-training developed through these investigations and parallel practice, the introduction of repetition maximum by DeLorme being one example. These concepts established as modern definitions of exercise variables enabling precise prescription of training loads for a variety of populations and training goals [18].

Although this development started after the Second World War, resistance training was not part of general exercise guidelines until much later. The American College of Sports Medicine (ACSM) position statement on exercise for healthy individuals from 1978 primarily concerned physical fitness in terms of cardio-respiratory fitness [46]. Since the updated 1990 ACSM statement, resistance training is recommended to be included as part of a sensible, general training program [47]. The introduction of resistance-training as part of the ACSM recommendation also coincides with specific recommendations on resistance training being part of

[1] In the current text, exercise is defined as an acute bout of physical activity designed to affect physical characteristics such as strength, speed or endurance. Training is defined as the systematic process of combining multiple exercise-sessions performed in sequence over time. DeLorme first used the adjective *heavy* to describe the resistance prescribed to overcome during exercises but later changed this adjective to *progressive*. In modern texts, the adjective is commonly omitted from the description and *resistance exercise/training* is used to describe strength-promoting exercises and training regimes requiring the neuromuscular system to exert force against resistance. Omitting the adjective has led to many heated debates among exercise physiologists as "endurance exercises are also performed against a[n] (external) resistance." With no ambition to resolve any conflict in the area, *resistance exercise/training* will be used synonymous with *progressive* or *heavy resistance exercise/training*.

other consensus statements [48, Ch. 2]. Consequently, informed by epidemiological data, the most recent general guidelines for physical activity do include resistance training [49].

It could be argued that the above account reflects the fact that common understandings of *why* and *how to* exercise are influenced by societal norms and historic events such as the search of national identity in the nineteenth century or war injuries in the twentieth century [30,33]. In attempting to outline contemporary influences on exercise prescription, one could argue that the development of techniques to collect large amount of biological data is one such influence. The continuously decreasing cost of a sequenced human genome [50] can serve as an example of this development. Such molecular techniques has enabled efforts to describe mechanisms by which exercise training induce favorable adaptations. The newly established Molecular Transducers of Physical Activity Consortium is an example of a large scale effort, explicitly initiated to develop personalized exercise recommendations and identify molecular targets through which effects of exercised may be mimicked [51]. Advances in bio-medical technologies are enablers of this enterprise and the quest to *individualize* exercise based on molecular diagnostics can be seen as a motivation for modern exercise science [51, 52]. Scientific research into contemporary exercise prescription can thus be understood as a part of the era of *personalized medicine*.

A challenge facing this program is to accurately describe etiologies of response heterogeneity associated with physical training. A wide variation of individual responses are commonly observed after standardized resistance-training programs where measures of muscle strength-changes varies from -32 to +250% and muscle size-changes varies from negative to (-11%) to impressively large (+59%) [53, 14]. By relating such variations to individuals genome (DNA) [54], and messenger RNA (mRNA) profiles [55, 56] we are beginning to gain knowledge about the genetic influence on training responses. In such studies, a common strategy has been to dichotomize responses into "responders" and "non-responders" to exercise training. From a public health perspective this is probably fruitful when *non-response* is defined as the absence of meaningful health-related adaptations, or even adverse effects in response to a given training regime [52, 57]. The existence of non-responders would have large implications regarding exercise prescription on the population scale [58], and for any given individual as it, if properly diagnosed, would guide clinical decision-making.

A key aspect of successful exercise diagnostics would be to take advantage of the relationship between exercise variables (i.e. modality, intensity, volume etc.)

and exercise response for a given individual. By adapting an individuals training program based on some prior knowledge about the individual, it is possible that the response could be positively affected. Observations supporting such notion exists as an individual classified as non-responsive to a specific exercise modality (e.g. endurance training) may be classified as a responder to another (e.g. resistance training) [59]. Even changing training variables within a specific modality have been shown to convert non-responders to responders as endurance training volume was increased [60].

Although apparent reversal of non-response to exercise training has been observed by manipulating training variables, decisive indications for such manipulations are still lacking.

2.2 Adaptations to resistance training

2.2.1 Muscle hypertrophy and strength

Systematic resistance training typically increases muscle mass and strength, adaptations through which many beneficial effects on health (e.g. increased amino acid storage physical independence) and athletic performance are conveyed. Muscle growth is a well characterized response to resistance training. On the whole muscle level, healthy untrained individuals can be expected to increases their muscle mass by $\sim$ 5-20% when training is conducted over two weeks to 6 months [14, 61, 53]. Over this time span, muscle growth is approximately linear with time [62, 63, 64] and has been detected as early as 3-4 weeks after training initiation, without apparent muscle edema [62, 63].

Relative muscle growth can be expected to be more pronounced in upper- compared to lower-body muscles when loading patterns are similar [61, 65]. This possibly relates to greater every day activity levels of lower-body muscles requiring a larger stimuli for adaptation [66]. Small, but detectable differences in muscle growth is typically seen between sexes for training induced muscle growth in the upper-body [53] but not for lower-body muscles [67]. Furthermore can hypertrophic responses be expected to be reduced with increasing age [65, 68] but increased with sufficient addition of dietary protein [69]. Additionally, training variables such as intensity, volume, frequency (reviewed below) together with other training aids, e.g. manipulation of blood flow through pressure cuffs [70] can effectively modulate resistance-training induced hypertrophy. Together, this underlines that both non-modifiable (e.g. sexual dimorphism and age) and modifiable factors (e.g. training

variables and protein supplementation) affects training-induced muscle hypertrophy.

Whole muscles growth in response to short term resistance training occurs primarily through growth of individual muscle fibers (muscle cells). This can be assumed as training-induced splitting of existing fibers and/or myogenesis, formation of new muscle cells, are likely slow processes and any increase in the number of fibers by such mechanisms would only represent a small addition to the whole muscle mass [71, 72, 73, 66]. Growth of muscle fibers transfers to greater muscle strength trough an expansion of the fibers contractile elements. The muscle cell is to a large degree occupied by myofibrils (about 80% of the cell volume [74]), which in turn contain sarcomeres, arranged in series. Upon neural activation and subsequent Ca^{2+} release, the sarcomere shortens through interaction between actin and myosin resulting in force generation [75]. With resistance training, the number of parallel myofibrils increases with the growth of individual fibers [76] leading to a greater force generating capacity of the whole muscle [74].

Measures of whole muscle size corresponds well with strength, particularly when a measure of muscle size reflects the muscles cross-sectional area [77, 78]. However, in response to resistance training, increases in strength are typically greater in magnitude than muscle growth [79, 80, 64, 81]. When relationships between resistance-training induced change in muscle size and strength in previously untrained individuals are analyzed, only a portion of the variation in muscle strength can be accounted for by changes in muscle size ($\sim$ 2.5-28%) [78, 14, 82] depending on type of measurements and statistical model used [82] This underlines that muscle hypertrophy is one contributing factor to muscle strength gains, but not the only one.

Different experimental models have shown that strength can increase without concomitant muscle hypertrophy. Getting acquainted to the actual strength test through repeated training of maximal performance produces similar gains in strength without concomitant hypertrophy [83]. If resistance training is performed unilaterally and the contralateral limb act as a control, strength gains are typically also seen here [84, 85]. Additionally, systematic imagery training, without muscle activation produces greater strength gains than control and low-intensity training conditions [86]. Together these observations indirectly points to the central nervous system and motor learning as important factors for strength gains. In addition to effects that mainly can be attributed to motor learning, resistance training leads to changed behavior of motor units, estimated from surface electromyograms [87]. Such changes could be attributed to morphological and functional changes of motoroneurons [88].

2.2.2 Changes in muscle fiber contractile and metabolic characteristics with resistance training

In adult human skeletal muscles, muscle fiber types can be identified based on their myosin heavy chain isoform composition. Pure fibers express a single mysoin heavy chain isoform whereas hybrid fibers co-express isoforms. Primarily three myosin heavy chain protein isoforms are expressed in adult human skeletal muscle, determined transcriptionally through expression of the genes *MYH7*, *MYH2* and *MYH1* corresponding to myosin heavy chain I, IIA and IIX [89]

Single fibers of different fiber types have specific contractile properties regardless of muscular origin [90], with type II fibers displaying greater force-generating capacity and shortening velocities than type I fibers when normalized to fiber cross sectional area [91, 90] These differences directly relates to the myosin heavy chain proteins displaying different characteristics when interacting with actin [92]. Importantly, *in vitro* assays performed at physiological temperatures shows that myosin heavy chain isoforms extracted from type II fibers are two-fold faster compared to type I fibers with no difference between type IIX and IIA [92].

In addition to contractile characteristics, fiber types identified on the basis of their myosin heavy chain content also differs in metabolic profiles. Type I fibers are characterized as having lower glycolytic but higher oxidative potential compared to type II fibers [93]]. Differences in metabolic profiles relates to fatigue resistance were type I fibers can maintain power output as well as ATP levels during intense exercise but type II and especially type IIX fibers fail to do so [89, 94].

Fiber type characteristics effectively modulate the muscle's ability to perform specific activities. Differences in fiber type composition within individuals, between different muscles, reflects this as anti-gravity muscle of the lower body typically express more type I fibers compared to upper-body muscles [89, 90]. Differences in fiber type composition between individuals and sexes are to some degree genetically determined [95, 96, 97]. Resistance training on the other hand is among non-genetic factors influencing fiber-type composition. Short term resistance training, designed for muscle hypertrophy and strength gains specifically converts type IIX fibers to more fatigue resistant type IIA fibers with unaltered type I fiber proportions [98, 99, 100, 101]. Such conversion is apparent both when measured on the protein and mRNA level [101, 100]. In contrast, reduced activity or inactivity readily increases the proportion of type IIX expressing fibers [102] [103]].

Concomitant with resistance training-induced muscle hypertrophy, and fiber type switch, the relative mitochondrial density of the muscle decrease as myofibrilar

protein fractions increases, evident from electron microscopy [74]. However, a single session of resistance training, albeit with low resistance (30% of 1RM), was shown to increase mitochondrial, as well as myofibrilar and sarcoplasmic protein synthesis rates. When exercise was performed with slower movement speeds (longer time under tension), the increase in mitochondrial protein synthesis was shown to be greater [104]. This indicates that the magnitude of metabolic stress induced by resistance training affects the subsequent mitochondrial remodeling. Such remodeling could explain improved mitochondrial function, measured as mitochondrial respiration in response to 12-weeks of resistance training with less pronounced changes seen in mitochondrial proteins [105]. An improved mitochondrial efficiency could also be linked to fiber type transitions. Mitochondria have the ability to form dynamic networks within cells by fusion (and fission) of individual mitochondria, a characteristic important for normal function [106]. Such behavior have been shown to be fiber type specific in muscle, with oxidative fibers (type I and IIA) compared to glycolytic fibers (type IIX and IIB in mice) displaying greater, elongated mitochondrial networks [107]. In response to endurance training, fiber type switch from glycolytic to oxidative, coincided with switch to less fragmented mitochondria [107]. Such coordinated remodeling can be linked to common molecular mechanisms regulating both fiber type and mitochondrial biogenesis [108, 109].

2.2.3 Connective tissue

In addition to adaptive changes to the contracting apparatus of single muscle fibers and neural mechanisms regulating their activity, resistance training modulates bone, tendon and connective tissue. From a general perspective, tissues enabling e.g. locomotion by conveying forces produced by contracting muscles and stabilizing body segments adapts in an activity-specific manner [110, 111] Specifically, short term resistance training leads to changes in mechanical properties of bone without increases in bone mineral content or density suggesting qualitative changes [112] Similarly, tendons respond to short term resistance training by increasing stiffness at high mechanical stress-levels without increased cross sectional area [113]. Interestingly, these adaptations seem to reach a plateau as no additional change in this characteristic is seen in individuals who have exercised over four years. However increased stiffness at lower mechanical stress levels have been documented [111]]. Changes in tendon properties in response to resistance training may thus primarily be associated with qualitative changes [111] as consequence of exercise and training-induced increased turnover of collagen in tendons, indicating

remodelling [111, 114].

Muscle fibers are embedded in connective tissue, observed as surrounding the whole muscle (epimysium), muscle fasicles (perimysium) and muscle fibers (endomysium) [115]. Connective tissue structures constituting the endomysium connects muscle fibers to adjacent fibers, capillaries and nerves which together with higher order structures make up the extracellular matrix enabeling mechanical and biochemical interaction between cell types [115, 116]. Together with the myotendinous junction, intramuscular structures (primarily perimysium) transmits forces originating from contracting muscle fibers to tendon and bone and act as an elastic energy storage during e.g. locomotion [116]. The mechanical properties of the extracellular matrix also allows for mechanical stimuli to be converted to biochemical signaling initiating e.g. responses to exercise.

There is a general coordination between connective tissue and muscle-cell remodeling in response to loading, evident coordinated responses from different cell types in response to exercise [116]. The major constituent of the extracellular matrix is collagen, produced in fibroblasts. In response to acute endurance-type exercise, collagen synthesis and muscle cell specific protein synthesis (myofibrillar and sarcoplasmic fractions) rise in a coordinated fashion [117]. Also, in response to short- and long-term resistance training with subsequent muscle hypertrophy, relative collagen content of muscle tissue remains stable [73]. However, fine tuning of such coordination could exist as contraction mode has shown to differentially affect myofibrillar protein but not collagen synthesis after acute exercise [118].

Increased remodeling of components of the extracellular matrix is a typical response to resistance training evident from gene expression studies [119, 120], and studies of acute protein synthesis [121, 118]. Such remodeling may contribute to increases in specific force (force generated per muscle cross section) seen after resistance training through improved lateral force transfer [122].

2.3 Effects of exercise prescription on muscle mass and strength

Precise exercise-training prescription gives information on exercises, their sequential order, intensity and volume, rest periods between efforts or sessions and the frequency at which exercise sessions are to be performed [24]. By manipulating these variables, resistance training programs can be tailored to better fit goals and starting points of any individual. The relative importance of exercise-training

variables for training outcomes has been examined in numerous studies including (but not limited to) the overall organization of exercise sessions, [123, 124] training frequency [125], and intensity [126]. It could be argued that training volume is of particular importance for muscle growth as when this variable is held constant, manipulation of other variables has little or no effect hypertrophy [127, 126]. For development of strength, factors such as intensity and within session organization of exercises is of importance [128, 129], however, when other factors are held constant, increased training volume generally leads to increased strength [128,130, 23], similarly to effects of training volume on muscle growth [24,25].

2.3.1 Effects of resistance exercise volume on muscle strength and mass

Exercise volume can be prescribed as the within session number of sets performed per muscle group. This unit is practical as it comparable between individuals and muscle groups [131]. Berger conducted an early study concerning effects of resistance exercise volume with the goal to determine what method most efficiently produced strength gains (in healthy young males) [132]. Berger compared one, two and three sets performed with two, six or ten repetition maximum (RM) in the bench press, three times per week, over twelve weeks. As the combined effect of three sets per session was superior regardless of the number of repetitions performed Berger concluded in favor of three sets. This conclusion was later challenged on the basis of data interpretation [27, 19]. Reveiwing the study by Berger and others, Carpinelli and Otto arrived to the conclusion that there was "insufficient evidence to support the prevalent belief that a greater volume of exercise (through multiple sets) will elicit superior muscular strength or hypertrophy" [27]. This stand has since been repeatedly put forward as a criticism of higher volume training programs [133,134] and sparked considerable scientific activity. The main argument against the recommendation of additional volume in strength training programs has been the lack of statistically significant results in single studies [19,133]. Indeed, individual studies do not generally agree on dose-dependent effects of training volume on muscle mass and strength gains [135, 136, 137, 138, 139, 140, 41, 141, 142, 143, 144, 145], including studies performed within participants, where different training volumes are allocated to either extremity [146, 147]. For example, differences in strength are between volume conditions are found in older individuals [135, 136, 41] but not confirmed in another study [139]]. Studies shows that more volume does not lead to increased muscle gains in young individuals [143, 141, 137]

a conclusion challenged by others [145, 138].

As previously noted, combining the above results and additional studies, meta-analyses concluded that training volume dose-dependency exists for the development of muscle mass and strength [[128]; [130]; [23]; [24,25]. As a second argument against additional volume in resistance training recommendation has been the cost/benefit relationship of adding training volume without meaningful or substantial additional gains [19, 133], a subsequent question is, whom would benefit from greater volumes and whom would not? Schoenfeld *et al.* combined data from published studies to explore if participant characteristics of the above mentioned studies interacted with training volume in explaining study outcomes. Neither sex, muscle groups nor age interacted with volume prescription indicating that no such factor would be able refine training prescription guidelines [25]. As the number of studies used to synthesis the meta-analysis was relatively low ($n = 15$) and the studies were heterogeneous in terms of e.g. outcome measurements, it may have lacked in power to detect any meaningful interactions. Additionally, included studies may not have been reporting relevant characteristics for such analysis.

Collectively, the available evidence suggest that there is overlap between training outcomes in studies were different volume has been utilized. The overlap cannot, with available data, be explained by general population characteristics such as age or sex. Studying the effect of different training volumes within participants could potentially help to define determinants of training outcomes in response to different volume conditions. Two within-participant studies have investigated the effects of training volume on strength and hypertrophy outcomes. Sooneste *et al.* compared strength outcomes in response to three- and one-set elbow flexor training for 12 weeks in young males using a whitin-participant protocol (arms allocated to either volume condition). The results showed general benefit of three- over one-set training for muscle hypertrophy and tended to do so also for strength gains [147]. No attempts were made to relate baseline characteristics to the magnitude of differences between volume conditions, presumably due to the small sample size ($n = 8$). Mitchell *et al.* compared muscle hypertrophy and strength gains in response to three- and one-set of knee-extension exercise performed three times per week for ten weeks. The study contained an additional training condition (low intensity, 30% of 1RM performed with three sets) with participants legs assigned to either of the three conditions in a random fashion. No significant differences were reported between volume conditions for muscle mass or strength gains [146]. However, the analyses were performed without taking the correlation between individuals into account due to the mixed design [146]. No attempts were made to relate any

measured characteristic to differences in responses.

2.4 Molecular determinants of training-induced muscle hypertrophy

Muscle mass fluctuates as a consequence of the balance between muscle protein synthesis and breakdown. When a net-positive balance is achieved, muscle protein accumulates and muscle mass increases. Following a single bout of resistance exercise, muscle protein synthesis increases over resting levels up to 48-h post-exercise [148, 149, 150, 151, 152, 153] after being blunted during exercise [148]. Muscle protein synthesis and breakdown rates are highly correlated [154, 150] indicating that these processes are mechanistically coupled and fluctuates together. While acute resistance exercise thus also stimulates to breakdown of muscle protein, it does so to a lesser extent, leading to an increase in the net protein balance from baseline under favorable conditions [154, 150, 155, 156]. When resistance training is performed under such favorable conditions, in the fed state with availability of dietary protein, a net positive protein balance can be expected after exercise [155, 156].

Indispensable for the process of protein synthesis is the ribosome, a cellular machine capable of translating genetic information in the form of messenger RNA (mRNA) to proteins. A synthesizing ribosome consists of four ribosomal RNA species (rRNA 18S, 5.8S, 28S and 5S) and about 80 proteins constructing two ribosomal subunits. Translation of mRNA occurs at the ribosomal core as ribosomal RNA catalyzes binding of amino acids to form a polypeptide chain corresponding to the ribosome-bound mRNA sequence. The rate of translation per ribosome (i.e. translational efficiency) can be modified by specific stimulus, of which mechanical stress and nutrients are of importance to this discussion. Mechanical stress (such as resistance exercise) or amino acid availability (as increased after ingestion of dietary protein) stimulates to protein synthesis through increased translational efficiency. This can be observed as the formation of polysomes as functional ribosomes bind to mRNA in response to e.g. mechanical stress [157, 158]. The available data from acute studies on protein synthesis in humans suggests that resistance training leads to muscle hypertrophy through the accumulative effect of repeated bouts of an anabolic stimuli. In recent years, this view has been supplemented by evidence suggesting that chronic resistance training also leads to increased rates protein synthesis at rest [153, 159, 160], which has been

postulated to be associated with an accumulation of ribosomes, i.e. an increased translational capacity [160, 161]. This notion is supported by training-induced increases in muscle RNA abundance. As the RNA pool to a large degree consists of ribosomal RNA [162, 163], total RNA can be used as a surrogate measure of ribosomal abundance.

Given the above discussion, the concepts of translational efficiency and capacity can be illustrated by data from Millward [164], wherein RNA concentrations and its association with protein synthesis rates were measured in rats starved or fed a diet containing protein. Protein feeding increased the rate of protein synthesis, but the relationship to RNA (ribosomal) abundance was largely maintained (Figure 2.1). This underlines the fundamental importance of ribosomal abundance and activity in determining protein synthesis.

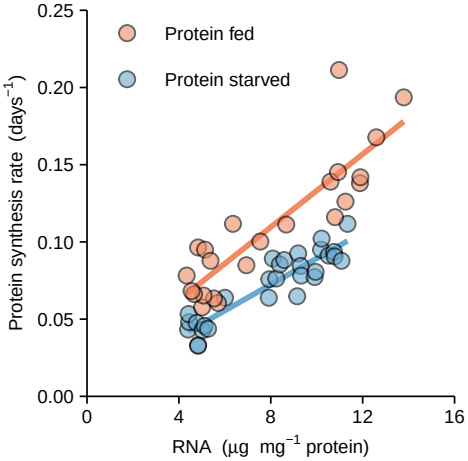


Figure 2.1: Relationship between RNA abundance and protein synthesis in rat skeletal muscle. Rats were either starved or fed a protein rich diet stimulating protein synthesis. Data from [164].

2.4.1 mTORC1 a multifaceted coordinator of cell growth

The discovery of an organic compound called rapamycin in the 1960's led to the characterization of a rapamycin sensitive protein involved in cell growth. The protein was later named mechanistic target for rapamycin (mTOR) [165]. mTOR is found in two protein complexes (mTOR complex 1, mTORC1; mTOR complex 2, mTORC2) where primarily mTORC1 has been found to be sensitive to rapamycin treatment [165]. Bodine *et al.* performed a comprehensive characterization of mTORC1-mediated skeletal muscle hypertrophy using rodent models, showing

that mTORC1 activation was essential for load-induced hypertrophy. Additionally, using transfection techniques, they showed that constitutively activated signaling upstream of mTORC1 (Akt) led to hypertrophy in an mTORC1-dependent manner, confirmed with concurrent administration of rapamycin [166]. Further utilizing rapamycin in genetically modified mice where mTOR was made rapamycin-resistant, specifically in skeletal muscle cells, confirmed that muscle fiber-specific rapamycin-sensitive mTORC1 signaling was needed to induce muscle hypertrophy in response to mechanical loading [167]. These mechanistic studies supports previous observational evidence connecting mTORC1 signaling to muscle growth in rats [157], and more recently, in humans [20, 168].

Administration of rapamycin in humans has also confirmed that mTORC1 signaling is important for protein synthesis in the acute phase after resistance training (2 hours) and in response to protein ingestion [169, 170]. However, extending the time-frame (up to 24 hours), differences in responses to resistance exercise between rapamycin treatment and control conditions were less pronounced [171]. This could in part be explained by the lower dosage of rapamycin administrated in human compared to animal trials but also indicate rapamycin-insensitive mechanisms controlling translational efficiency [172, 173].

mTORC1 functions as a signaling hub by integrating multiple environmental cues to regulate cellular growth. Among such cues is mechanical stimulation which leads to accumulation of phosphatidic acid in muscle cells [174]. Such accumulation was shown to be independent of mechanisms upstream of mTORC1 (phosphoinositide 3-kinase (PI3K)/AKT and ERK signaling) [174, 175] but still readily led to mTORC1 activation [174, 175]. In cellular models, phosphatidic acid has been shown to interact with mTORC1 on the same site that is targeted by rapamycin [176] indicating a more direct link between mechanical stimulation and mTORC1 activity. The enzyme primarily responsible for mechanically induced increases of phosphatidic acid has been shown to be diacylglycerol kinase ζ [177].

In the context of muscle growth in response to resistance training, adequate supplementation of dietary protein augments responses [69]. Mechanistically, dietary protein intake increase the availability of amino acids in muscle cells and these in turn stimulate protein synthesis through mTORC1 by multiple mechanisms [178]. mTORC1 capabilities to fine tune its response based on cellular status can be exemplified from studies examining responses to different amino acid compositions. Providing a mixture of essential amino acids potentiated mTORC1 signaling in response to resistance exercise more than provision of essential amino acids without leucin or leucin alone [179].

In addition to mechanical stimuli and amino acids, mTORC1 integrate several environmental cues related to growth factors, energy and oxygen status with downstream signaling differing depending on upstream signaling and cellular characteristics [180]. Two downstream targets of mTORC1 rely much of the information related to translational control one of which is the well characterized eIF-4E (eukaryotic translation initiation factor 4E)-bining protein 1 (4E-BP1). Upon activation, 4E-BP1 releases eIF-4E [181] which enables formation of an preinitiation complex and subsequent recruitment of the small ribosomal subunit to mRNA [182]. eEIF-4E-dependent initiation of translation is believed to be rate limiting and thus a control point for protein synthesis. Interestingly, formation of the preinitiation complex, induced by mTORC1-4E-BP1-mediated release of eIF-4E results in enhanced translation of special classes of mRNAs, containing a 5' structures that does not permit efficient translation [182,183] Among the resulting gene products from such mRNA are growth factors cell cycle regulators such as cyclin D1 and c-Myc as well as ribosomal proteins [165,182].

Parallell to 4E-BP1, is another well described downstream target of mTORC1, S6 kinase 1 (S6K1). S6K1 was named after its ability to phosphorylate ribosomal protein S6, but has since been shown to have multiple roles related to both translational efficiency and indirectly to translational capacity [165]]. The importance of S6K1 in control of muscle mass is apparent from S6K1 depletion in mice that results in reduces muscle growth and experiments showing that constitutevly active S6K1 resulting in increased myotube growth in cell cultures [184, 185]. S6K1 deficient mice did however not show a reduced translation of 5'TOP mRNA, an effect still sensitive to rapamycin [186]. Instead, S6K1 deficient mice have been shown to be unable to induce transcription of genes related to ribosomal biogenesis [187]. Upon activation of Akt, such mice fail to respond with increased ribosomal biogenesis, estimated through accumulation of total RNA and confirmed by targeted rRNA analysis [188]. S6K1 activity also leads to phosphorylation of downstream targets that enables translation initiation and elongation in addition to its most well known substrate ribosomal protein S6 (rpS6) [189]. Although a target of mTORC1, rpS6 have been shown to have conterintuitive role in protein synthesis. Despite its location within the ribosome close to its core, mice genetically modified to be unable to phosphorylate rpS6 upon stimulation still form polysomes indicating that rpS6 phsophorylation is not needed for translational initiation [190]. Protein synthesis rates in the same mice are also higher compared to wild type mice suggesting an inhibitory role of rpS6 phosphorylation in protein synthesis [190]. Interestingly, mice depleted of S6K1 showed reduced specific force compared to wild-type mice,

coinciding with forming of protein aggregates [188] Together these observations points to fine-tuning mechanisms in the S6K1-rpS6-axis, balancing protein synthesis, protein quality and energy wastage [188, 190]. Fine-tuning may also exist within the mTORC1-S6K1 axis as S6K1 stimulates to mTOR phsophorylation at Ser^{S2448} [191], a commonly used read-out for mTORC1 activity [192].

By unknown mechanisms, mTORC1 signaling is sensitive to training status evident from changes in acute signaling in response to resistance training depending on the acute training status [189, 193].

2.4.2 Ribosomal biogenesis and resistance training induced muscle hypertrophy

The overall role of ribosomal abundance in determining protein synthesis and subsequent cellular and tissue size was briefly mentioned above. In addition to correlations between RNA abundance and protein synthesis in mice [164] and cell culture [194] inhibition of ribosomal RNA (rRNA) transcription or inhibition of up-stream transcription factors act to diminish muscle cell growth upon stimulation [195,194,196]. In the context of resistance training-induced muscle growth, observational evidence from human studies further supports a determining role of ribosomal biogenesis to achieve increased translational capacity and enable hypertrophy. Figueiredo *et al.* observed a correlation between the changes in RNA abundance and magnitude of muscle growth over eight weeks of resistance training [197]. Stec and collegues observed increased ribosomal RNA and total RNA abundance only in participants that were classified as modest or extreme responders in terms muscle growth but not low responders after four weeks of resistance training [195]. Similarly, Mobley *et al.* found larger increases in total RNA in participants classified as high- vs. low-responders to 12 week resistance training (34% vs. 8% increase in total RNA) together with a correlation between total RNA increases and muscle growth over the same time period [198]. Together these studies underlines the importance of ribosomal biogenesis and translational capacity in resistance training-induced muscle hypertrophy. It should be noted however, that protein synthesis and cellular growth may occur in the absence of ribosomal biogenesis. In cultured myotubes stimulated with IGF-1, inhibition of ribosomal RNA transcription led to reduced RNA content but not myotube size compared to non-inhibited controls [199]. In aged male participants, three sessions of resistance training did not lead to increased levels of RNA but a 30% increase in protein synthesis rates [200]. Furthermore, in response to a greater

number of training sessions, absence or reduced ribosomal biogenesis are observed in selected individuals but muscle growth may still be detected [193]. However, when e.g. comparing aged and young skeletal muscle, aged muscle typically respond with reduced hypertrophy after mechanical stimuli, coinciding with reduced ribosomal biogenesis [193,201], indicating that potent ribosomal biogenesis is needed to support greater hypertrophy. Similarly, when comparing cell culture experiments, a "broader" stimuli induced by serum compared to a single growth factor could induce greater cellular growth that subsequently require support of an increased translational capacity [199, 196, 195].

Synthesis of ribosomes is indeed a hallmark of the early response to resistance training as a single resistance-exercise session leads to increases in precursor rRNA (pre-rRNA 45S) [202,203] and repeated bouts lead to accumulation of rRNA and thus presumably functional ribosomes [202, 204, 195,197, 193, 160]. However, a time course of ribosomal transcription and accumulation in response to resistance training in humans remains largely unstudied. Only a few studies have investigated exercise- or training-induced changes in markers of ribosomal abundance over multiple time-points. For example, two consecutive bouts of electrically evoked muscle contractions were associated with increased levels of total RNA, with peak values being observed 72 hrs after the second bout [204]. Using voluntary contractions, peak values in total RNA were reported after nine sessions, followed by a slight decrease to after 18 sessions [193]. These data suggest that ribosomes accumulates with some delay from initiation of training, reaches a plateau in the early phase of resistance training (three weeks) and slightly decreases as muscle mass further increases [193,204].

Synthesis of new ribosomes is a complex and energy demanding process. It involves the synthesis of both ribosomal proteins and four mature rRNA species as well as their assembly into functional ribosomal subunits [205, 206,207]. All mature rRNA (except rRNA 5S) are derieved from a single pre-rRNA transcript (45S pre-rRNA). After being transcribed from ribosomal DNA (rDNA), the 45S pre-rRNA transcript goes through several "splitting" events ultimately leading to the formation of three rRNA species, 18S, 5.8S and 28S. Coinciding with step-wise splitting of the pre-cursor transcript, modifications to the rRNA structure and assembly with ribosomal proteins into precursor ribosomal subunits occur in the nucleolar compartment [206]. After export to the cytoplasm, additional maturation steps are required in both subunits before they can form functional ribosomes [206].

Ribosomal biogenesis is believed to be determined by the rates of pre-rRNA

transcription by RNA polymerase I (Pol I) which in turn is regulated by coordinated assembly of a complex of transcription factors at the rDNA promoter [207].. This process is co-regulated with translation as initiation of rDNA transcription is controlled by e.g. mTORC1 through multiple mechanisms. Activation of the of the upstream binding factor (UBF) through phosphorylation is needed to initiate transcription [208, 209], and such activation is partly controlled by mTORC1 activity, with its inhibition being associated with blocked UBF phosphorylation and reduced UBF availability trough de-phosphorylation of retinablastoma leading to reduced ability of UBF to recruit secondary factors to the rDNA promotor and stimulate rRNA transcription [210, 211]. mTORC1 also controls one of these secondary factors, TIF-1A, as rapamycin leads to specific phosphorylation and translocation away from the nuclei [212].

Parallell to mTORC1 control, specific inhibition of MEK showed that MEK/ERK signaling is important for UBF binding to rDNA [213]. Furthermore, the transcription factor c-Myc has also been implicated in ribosomal biogenesis as its inhibition coincides with less UBF activity and rRNA transcription irrespective of mTORC1 signaling [194]. c-Myc is also found at the rDNA promotor and is required for rDNA transcription [214, 215], in addition to its role in transcription of genes imoportant for rRNA transcription, e.g. UBF [216] Interestingly, the availability of UBF *per se* has also been shown to be a determinant of rRNA transcription [217, 211] through control of rDNA gene activity [218].

In summary, ribosomal biogensis as well as the regulation of translation is under coordinated control of several pathways integrating multiple stressors and environmental cues to regulate cellular protein synthesis.

2.4.3 Transcriptional regulation of training-induced muscle remodeling

[219]

[220]

[221]

2.5 Effects of exercise volume on molecular determinants of muscle growth

Given that exercise-training variables can modify responses to resistance training, it is reasonable to assume that these effects are mediated through determinants

of muscle growth. Exercise intensity has been evaluated with respect to protein synthesis and activation of targets downstream of mTORC1 (4E-BP1 and S6K1). Kumar *et al.* showed that maximal stimulation of fractional synthetic rate was achieved with intensities greater than 60% of 1RM, coinciding with signaling events [222]. The same group subsequently investigated the effect of training volume at an intensity presumably leading to maximal stimulation of protein synthesis (75% of 1RM). This analysis revealed a volume-dependent dose-response as six-sets of leg extension led to greater protein synthesis one hour after exercise and sustained S6K1 phsophorylation over up to four hours after exercise [223]. Further extending the time frame, Burd and colleagues evaluated a single session consisting of either one or three sets with biopsies sampled 5, 24 and 29 hour after exercise. Both conditions led to increased myofibrillar protein synthesis five and 29 hours after exercise, but to a larger extent in response to three sets. However, volume-dependent regulation of S6K1 was only seen at 29 hours after exercise with earlier events ($<$ 5 hours) possibly missed due to the timing of biopsy sampling. No clear volume-dependency was seen in p90RSK1 (downstream of ERK) or rpS6, however eukaryotic initiation factor 2B (eIF2Bϵ) phosphorylation was reduced only in the three set condition at five hours post exercise [20], presumably mediating translation initiation, although its exact role is still unclear [224]. Volume dependent regulation of S6K1 at Thr389 and rpS6 at Ser235/236 were reported 30 minutes after exercise by Terzis *et al.* as six sets of 6RM bilateral legpress resulted in greater phosphorylation compared to three and one set [21]. No clear differences between volume conditions were seen in Akt at Ser473, mTOR at Ser2448, ERK 1/2 at Thr202/Tyr204, p38 (α,α and δ) at Thr180/Tyr182, p38γ Thr180/Tyr182 or AMPK at Thr172 [21]. Corroborating previous observations regarding exercise volume-dependence of the S6K1-rpS6-axis, Ahtiainen and colleagues also found greater phosphorylation of S6K1 at Thr389, rpS6 at Ser235/236 and Ser240/244 30 minutes after exercise with ten compared to five sets of 10RM legpress. Furthermore, ERK1/2 at Thr202/Tyr204 and p38 at Thr180/Tyr182 phosphorylations were not found to be volume dependent [22]. However, in contrast to Terzis *et al.*, they reported volume-dependence in exercise-induced phsophorylation of AMPKα at Thr172, this together with AS160 at Thr642, IRS-I Ser636/639 and Akt at Ser473 together with a tendency in LKB1 at Ser428, all related to cellular energy status, glucose uptake and metabolism [22,225]

Collectively, although limited in precision due to small sample sizes (n = 8-19) and temporal resolution and these studies points to volume dependency in exercise-induced S6K1$^{\mathrm{Thr389}}$ phosphorylation, potentially further augmented when increased

number of sets are examined in the acute phase (> 3 sets, < 5 hours; [20,146] vs. [21,22,223]). Increased S6K1 phosophorylation coincides with rpS6$^{Ser235/236}$ and $^{Ser240/244}$ in the acute phase (< 5 hours) after exercise [21,22] but not in the late phase (29 hours) [20]. Although rpS6$^{Ser235/236}$ is a known substrate for both S6K1 and ERK1/2 and p90RSK1 [226], ERK1/2$^{Thr202/Tyr204}$ was not been found to be volume-sensitive in the acute phase [21,22], nor was P90RSK1^{Thr573} in the late phase [20] suggesting that signaling along the mTORC1-S6K1-rpS6-axis is more sensitive to variable volume than the parallell MEK/ERK-axis. The above signaling events suggest exercise volume-dependent activation of the translational machinery, and increased volume indeed leads to increased muscle protein synthesis [20,223]. However, relatively high exercise volumes may lead to signaling events inhibiting protein synthesis as AMPK^{Thr172} showed volume dependency, presumably relating to cellular energy stress and glucose metabolism [22,225, 227]. Interestingly, an increased AMPK-related signaling may be related to an accumulated effect due to short inter-set rest periods, coinciding with reduced proteins synthesis, at least in the acute phase after exercise [228].

In addition to signaling events and , training-induced lead to increased satellite cells [229]

2.5.1 From Training response

In humans, the biological adaptation to resistance training varies with exercise-training variables such as volume, intensity, rest between repetitions and sets, selection and order of exercises, repetition velocity and frequency of training sessions [17]. In addition, genetic and epigenetic disposition and environmental factors play a role in variations in adaptations [58, 230, 69]. As time constraints often hinder participation in exercise training-programs [26], numerous studies have searched for the minimal required exercise dose to promote beneficial adaptations. Within-session volume has received particular attention, and although a handful of studies have shown that low-volume training provides similar gains in strength and muscular mass as moderate-volume training [144, 142, 146], meta-analyses conclude in favor of moderate volume protocols [23–25,231]. This apparent discrepancy of specific studies to demonstrate benefits of increased training volume is likely due to a combination of small sample sizes and substantial variation in training responses between individuals and experimental groups. In theory, within-participant designs should alleviate these limitations.

2.6 From RNA-seq

Skeletal muscle is a highly adaptable tissue that responds to environmental stress by altering growth rates and differentiation processes. During resistance training, signaling cascades that stimulate muscle plasticity are triggered. Upon repeated exposures, this facilitates growth and a phenotypic shift in a metabolically active direction [232], with the opposite happening during inactivity [233]. Despite this generalized view, muscle responsiveness and plasticity vary, both in response to different resistance-training protocols [234] and, perhaps more importantly, between individuals [53,> 14]. Selected individuals show a near-complete absence of muscle growth after prolonged resistance training, which markedly reduces the beneficial outcomes of such interventions for muscle function and overall health [14, 53]. Currently, little is known about the etiology of this variation. However, it is usually associated with phenotypic traits of skeletal muscle [235, 195, 236], which implies interactions with environmental factors, genetics, epigenetics, and composites of the intra physiological milieu [69, 237]. This multifaceted origin makes the training-response-spectrum difficult to study directly, with each of the underlying factors offering limited explanatory value alone [58]. Instead, a more indirect approach is necessary, whereby the combined effects of the factors are targeted by studying global patterns of mRNA, protein expression, and skeletal muscle biology.

Previous studies have investigated transcriptome responses to acute resistance exercise [238, 119, 239] and chronic resistance training [240, 241, 119, 242, 238, 120], as well as described associations between transcriptome characteristics and degrees of muscle growth [120, 243], and function [244, 245]. Whereas these studies have merited interesting findings, they lack clear coherences in terms of differential expression events, even for classical exercise-inducible genes such as PGC1α [246]. This lack of clear coherence is potentially due to a combination of issues such as differences in study design and methods for synthesis and analysis of transcriptome data. First, biologically founded variability can be attributed to differences in exercise protocols (e.g., differences in exercise-volume or intensity). This makes it difficult to discern a general transcriptome exercise response, as training variables are not standardized between studies. Biological heterogeneity is also caused by differences between research participants, affecting signal-to-noise ratios and making it difficult to discern the effects of single independent factors such as training variables. Design stage decisions such as the use of within-participant designs [234, 247] are likely to reduce this variation and to provide transcriptome data with increased biological meaningfulness. Second, technical variability can be attributed

to decisions made during the bioinformatical treatment of data. As described by Concea *et al.*, there is no optimal pipeline for sequencing technology as new tools keep evolving and emerging, different tools should be explored to an optimum pipeline for the specific type of data [248]. To exploit the potential of any study design, there is a need for identifying an appropriate pipeline for transcriptome analyses to ensure a biologically valid interpretation of data. This entails identifying potential violations of common assumptions caused by the experimental model at hand, relating to, for example, data normalization [249, 250].

For transcriptome data to provide adequate biological information about a given experimental set-up, numerous bioinformatic steps need to be adopted in a customized manner [248, 251]. Of these steps, data normalization is particularly decisive [250], as it aims to transform naïve transcript counts into biologically meaningful results. This essentially means expressing them as *per*-cell abundances [252]. For most experimental models, this is equivalent to providing transcript-to-total RNA ratios, given the fulfillment of the assumption that total RNA levels remain stable between conditions on a per-unit-cell or per-unit-tissue basis [252]. In cell models that exhibit high degrees of plasticity, gene expression events result in increased amounts of total RNA and mRNA transcripts per cell [253], specifically violating the assumption that most genes are not differentially expressed [252, 249]. We are not aware of any study that has addressed the need to account for such perspectives during transcriptome analyses of skeletal muscle subjected to mechanical stress, such as resistance training. Indeed, this assumption can be expected to be violated, as total RNA content increases markedly on a per-unit-weight basis [234], with potential global changes also occurring for the mRNA pool, though this remains unknown. The extent to which total RNA, and therefore ribosomal RNA, increases, coincides with the increase in muscle mass [234,195], underlining its importance for cellular growth but also its inevitable presence as a potential confounding factor in RNA sequencing experiments.

3. Aims

The primary aim of this thesis was to relate the adaptive response to resistance training with low- and moderate-volume to skeletal-muscle characteristics in previously untrained individuals. The key question was whether manipulation of exercise-volume will have diverse effects in different individuals related to muscular intrinsic characteristics. A further aim was to characterize exercise-volume dependence in muscle molecular characteristics and determine a time course profile of markers of ribosomal biogenesis in response to resistance training. Based on these aims, the objectives of the present thesis were;

- to relate skeletal muscle and systemic characteristics to benefit of moderate- compared to low-volume resistance training;
- To determine volume-dependence in molecular networks related to muscle growth and remodeling in response to resistance training
- To determine a time course of markers related to ribosome biogenesis in the early phase of resistance training.

4. Methods

4.1 Study protocols and participants

Study I was designed to examine effects of low- (single-set per exercise) and moderate-volume (three sets per exercise, multiple-sets) on responses to acute exercise and long-term training within participants. A schematic overview of Study I can be seen in Figure 4.1. Prior to the training intervention, participants reported to the laboratory for bilateral baseline muscle biopsies (*m. vastus lateralis*). Bilateral muscle biopsies were additionally sampled prior to, and 60 minutes after the fifth training session as well as after the intervention (Figure 4.1). Assessments of muscle strength (isokinetic and isometric knee extension torque as well as one repetition maximum) were performed twice at baseline and further measured in weeks 3, 5, 9 and after the intervention (Figure 4.1). Body composition measurements (magnetic resonance imaging and dual-energy X-ray absorptiometry) were performed at baseline and after the intervention.

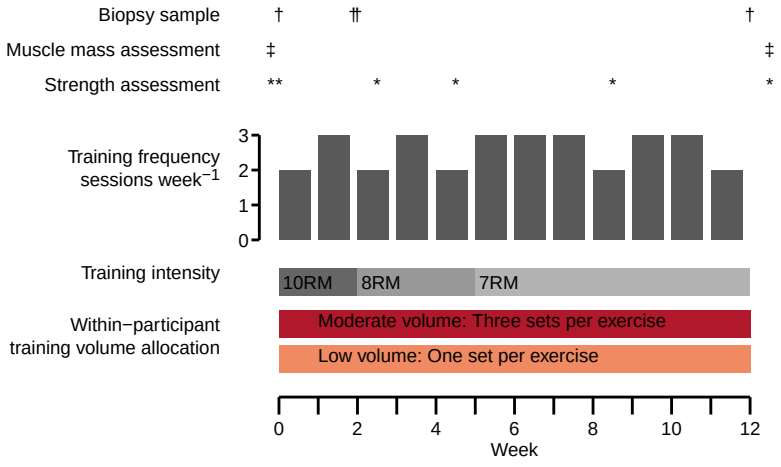


Figure 4.1: Schematic representation of Study I, see text for details.

Study II was designed to study the effects of resistance training *per se* and effects of variable compared to constant inter-session volume on selected markers related to ribosome biogenesis. Participants were therefore recruited to an experimental group ($n = 11$) and a non-training control group ($n = 8$). An overview of Study II can be seen in Figure 4.2. Baseline muscle strength (isokinetic and isometric knee-extension torque) was assessed during three initial visits to the laboratory, with the last baseline-assessment performed at least seven days prior to the first biopsy sampling. Follow-up measures of muscle strength in the experimental group were completed three and nine days after the last training session. Muscle thickness (*m. vastus lateralis*) was measured bilaterally prior to the study as well as two and eight days after the last training session in the experimental group. Muscle biopsies were sampled bilaterally prior to any training, 48 hours after the first, fourth, fifth, eighth, ninth and twelfth session as well as eight days after the twelfth session (Figure 4.2). The control group in Study II performed the same initial assessments as the experimental group. Muscle thickness was again assessed after a control period of 2-4 weeks. Follow-up muscle biopsies were sampled from one leg 48 hours after the first biopsy as well as after the control period. Follow-up strength assessments were performed 24 hours after the last biopsy.

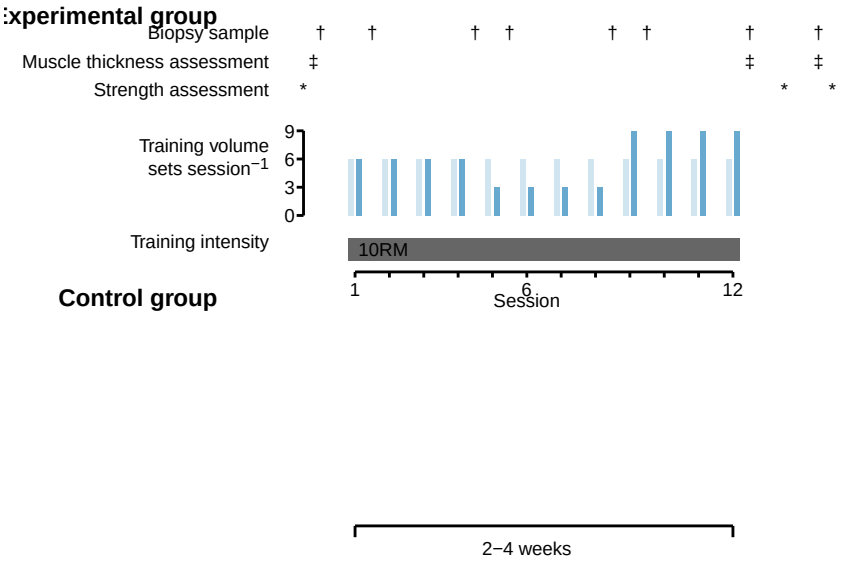


Figure 4.2: Schematic representation of Study II, see text for details.

Recruitment to both studies was done through advertising and word of mouth,

primarily at Lillehammer University College/Inland University of Applied Sciences. Potential participants were interviewed and matched against inclusion and exclusion criteria. During initial interviews, participants were informed of the study design and time requirements. Participants were also informed about potential risks and sources of discomfort associated with the study prior to giving their informed consent.

To be eligible for participation in both studies, participants had to be young (Study I age 18-40; Study II 18-35) and non-smoking. Both men and women were considered for participation. Exclusion criteria included a training history of more than one weekly session during the last 12 (Study I) or six (Study II) months leading up to the study. Participants were also screened for intolerance to local anesthetic, current or previous injuries affecting their ability to perform resistance training, self-reported symptoms or history of disease, intake of medications or supplements with known effects on adaptations to training. Participant characteristics for both studies are shown in Table 4.1.

Forty-one healthy individuals were recruited and 34 of these completed at least 85% of the prescribed sessions and were thus included in subsequent data analyses. Reasons for not completing the trial included injury not related to the study ($n = 1$), pain or discomfort during exercises ($n = 5$) and non-adherence to the study protocol. There were no systematic differences in characteristics between participants included in or excluded from data analysis in Study I.

Prior to the study, all participants reported that they previously had been engaged in sporting activities. At enrollment, twenty participants reported regular physical activities ranging from once every other week to four times per week. Ten participants reported performing resistance-type exercise at the time of enrollment limited to no more than once a week.

4.2 Resistance training interventions

Each training session started with a light, standardized warm-up (5 min ergometer cycling and 10 repetitions each of push-ups, sit-ups, back-extensions and squats). Before each exercise in the main program, one set of 10 repetitions were performed in the specific exercise with approximately 50% of 1RM.

Studies were fully or partially performed as within-participant studies as each participant had their legs assigned to different training conditions (not including the control group in Study II). Allocation was performed after enrollment where each participant had their legs randomized to either low- or moderate volume

Table 4.1: Participant characteristics

		Sex	Age (years)	Stature (cm)	Mass (kg)	Fat mass (%)	Lean mass (%)
Study I	Included	Female	22.0 (1.3)	168 (7)	64.4 (10.4)	34.1 (5.6)	64.3 (6.2)
		Male	23.6 (4.1)	183 (6)	75.8 (10.7)	20.4 (6.0)	79.3 (5.0)
	Excluded	Female	22.9 (1.6)	166 (8)	64.6 (9.7)	28.8 (8.7)	68.6 (9.1)
		Male	24.3 (1.5)	189 (5)	88.2 (22.4)	24.3 (15.3)	76.8 (12.7)
Study II	Training	Female	23.4 (2.9)	168 (8)	64.0 (9.2)	30.8 (7.1)	65.5 (6.8)
		Male	25.7 (5.8)	177 (3)	77.5 (8.0)	25.3 (3.9)	71.3 (2.4)
	Control	Female	24.1 (3.5)	166 (4)	63.8 (0.6)	30.5 (6.4)	66.3 (5.2)
		Male	25.5 (5.5)	182 (5)	76.5 (7.7)	18.2 (5.1)	78.7 (4.2)

Data are means and (SD)

(Study I, see Figure 4.1), or variable or constant volume (Study II).

In Study I, the low-volume protocol consisted of a single set of each exercise and the moderate-volume consisted of three sets per exercise. Three unilateral leg exercises were used (leg press, leg curl and knee extension). The moderate volume-leg commenced all sessions and the low volume-leg performed a single set of each exercise in the rest between second and third set of the moderate volume training protocol.

In Study II, only unilateral knee-extension was performed in an effort to concentrate the stimulus to the quadriceps muscles. The constant-volume leg performed six sets of 10RM throughout the study and variable leg performed six sets in session one to four, three sets in session five to eight and nine sets in session nine to twelve with same relative intensity (see Figure 4.2).

4.3 Muscle strength assessments

4.3.1 Isokinetic and isometric maximal torque

Maximal isokinetic and isometric unilateral knee-extension strength was determined by use of a dynamometer (Study I: Cybex 6000, Cybex International, Medway USA; Study II: Humac Norm, CSMi, Stoughton, MA, USA). After a brief warm-up (5 min ergometer cycling, RPE 12-14), participants were secured at the hip and shoulders in the dynamometer with the knee joint aligned to its rotation axis. Individual settings were recorded and used in subsequent measurements. Participants were familiarized with the test protocol by performing three sub-maximal efforts at each angular speed. In Study I, three angular velocities were used to determine

isokinetic torque (60°, 120° and 240° $\times$ sec^{-1}), in Study II an angular velocity of 90° $\times$ sec^{-1} was used for this purpose. In Study I, participants performed two attempts at 60° $\times$ sec^{-1} and three attempts at 120 and 240° $\times$ sec^{-1}. In Study II, three attempts were made at the designated angular velocity. In both studies, attempts within each angular speed were performed in immediate succession. After isokinetic testing, the lever was fixed at 30° (full extension = 90°), participants were instructed push with maximal effort for 5 seconds and the maximal isometric torque was recorded. Two attempts were made for maximal isometric torque in Study I and a single attempt was made in Study II. Sixty seconds of restitution was given between each measurement in both studies with the exception of between isometric contractions in Study I where a 30 second restitution period was used.

In subsequent assessment sessions, the first measurement was performed on alternate legs. In Study II, the dynamometer allowed for participants to remain seated for assessments of both legs. This was not possible in Study I as the measurement system required participants to be re-seated for assessment of the contra-lateral leg.

4.3.2 One-repetition maximum

One repetition-maximum (1RM) was assessed in unilateral leg-press and knee-extension in Study I. Each exercise was assessed after a specific warm-up (ten, six and three repetitions at 50, 75 and 85% of the anticipated maximum). Attempts were made with increasing resistance (four to six attempts) and one repetition maximum was defined as the highest resistance successfully lifted through the full range of motion.

4.3.3 Strength assessment frequency (and statistics)

In Study I, maximal values from each assessment and time-point was used in statistical analyses including two separate assessments at baseline, separated by at least four days. Strength was determined

In Study II, the maximal value from all successful attempts were used in statistical analyses with unsuccessful attempts identified based on obvious misinterpretation of instructions or failure to reach maximal subjective effort.
Strength assessments were separated by at least 48 hours from preceding training sessions.

At baseline, 1RM, isokinetic and isometric strength assessments were performed twice, separated by at least four days. The maximum value achieved for each of

the tests was used in subsequent analysis. Strength tests were separated by at least 48 hours from preceeding training sessions. A combined measure of muscle strength was calculated as the average of all tests (1RM, isometric and isokinetic), wherein each test modality was given equal weight. A subset of the participants (n=18) performed strength assessment during the course of the study (at week two, five and nine). For the remaining participants, ordinary training sessions were prioritised when participants missed training or testing due to illness or scheduling difficulties.

Muscle strength was assessed as maximal voluntary isokinetic (90° sec-1) and isometric (60° angle, fully extended leg 0°) knee extension torque. After a brief warm-up (5-min cycling, RPE 12-14), participants were seated and secured in the individually adjusted dynamometer. Participants were instructed to gradually increase their effort during three warm-up repetitions (50, 60 and, 70% of subjective maximal effort). After a 30-sec restitution period participants were instructed to perform three repetitions with maximal effort in the concentric phase. Sixty seconds after the isokinetic test the lever automatically moved to a 60° angle and participants were instructed to push against the lever enough to see feedback from the visual feedback system. After an additional 15-sec restitution period, participants were instructed to push against the lever with maximal effort. Within the same assessment session, participants remained seated in the dynamometer for measurement perfomed on both legs. The first measurement was alternated between legs every other session. For statistical treatment of the data, all successful attempts were used. The last strength assessment at baseline was performed at least seven days prior to the first biopsy sampling. At least one of the baseline strength tests was performed on separate day with two sessions allowed to be perform on the same day with a short rest between assessments. Post training assessments were performed 48 hours and eight days after the last session.

4.4 Measures of muscle mass

In Study I muscle mass was measured by magnetic resonance imaging (MRI) and dual energy X-ray absorptiometry (DXA) prior to and after the intervention. Both MRI and DXA measurements were completed during the same visit to the laboratory. Participants were instructed to refrain from strenuous physical activity during the last 48 h leading up to the measurements. The post-training measurements were completed at least 48 h after the last strength testing session. Participants were asked to refrain from food consumption during 2 h leading up to

the measurements.

MRI images were obtained from the mid-thigh and analyzed by the same investigator blinded for time (pre- and post-training) and condition (low- and moderate-volume). Multiple images were used to estimate the cross-sectional area of the extensor muscles at the same distance from the knee-joint.

See figure

In Study II, m. vastus lateralis muscle thickness was measured by B-mode ultrasonography (SmartUs EXT-1M, Telemed, Vilnius, Lithuania) using a 39 mm 12 MHz, linear array probe. At least three images were captured for each leg per time-point. Between each image acquisition the probe was relocated to the same position where on the skin and subsequently marked on a soft transparent plastic sheet superimposed on the thigh.

Muscle thickness of m. vastus lateralis and m. rectus femoris were measured using Transverse images were obtained $\sim$ 60 % distally from the trochanter major towards the femoral lateral epicondyle. Landmarks such as moles and scars were also marked on the plastic sheet for relocation of the scanned areas during post-training measurements. During analysis, pre and post images from the same participant were analyzed consecutively using the Fiji software,66 and by two independent researchers. The average muscle thickness of the three images captured per muscle was used for further analyses.

For determination of body composition participants were scanned using DXA (Lunar Prodigy densitometer, GE Healthcare, Madison, WI, USA) with the standard scanning mode (13-25 cm). Participants were lying supine within the scanning bed reference lines, with a strap secured around the ankles to ensure a standardized body position in each scan. The scans were conducted with participants in a fasted state between 07.00-10.00 AM, with empty bladder and wearing only under-wear. Prior to each scan, a phantom scan was run to prevent baseline drifting from affecting analyses. The same technician was used at each time point. Analyses was performed using GE enCORE version 17.0 software (GE Healthcare). Region of interest was customized for covering upper thigh, marked with a sqaure from pubic symphysis to lateral part of tuberculum major, and distal to art. genu.

Muscle thickness (MT) was measured using a B-mode ultra sound unit (SmartUS EXT-1M, Telemed, Vilnius, Lithuania). Participants lay supine in a relaxed position for 20 min before assessments, with their feet strapped in a standardized position. A mark was set on the line 60% of the distance between Spinia Iliac Anterior Superior and the lateral femur condyle. MT of *m.vastus lateralis* was measured applying a water-soluble transmission gel (Aquasonic 100 Ultrasound Transmission

Gel; Parker Laboratories Inc., Fairfield, NJ, USA), and a 39 mm 12 MHz ultrasound probe was placed perpendicular to the site of interest without pressing the skin. When the quality of the image was satisfactory, evident as distinct upper and lower muscle fascia, three images were captured, where the probe was relocated to the same position between each image. Position of the probe was marked on the skin and subsequently marked on a transparent paper to ensure similar probe placement for both the right and left *m.vastus lateralis* at subsequent assessments. Analyses were done in ImageJ Fiji [254] with images cropped and coded to ensure blinding of the assessor.

4.5 Muscle tissue sampling and preparations for downstream analyses

Muscle samples were obtained under local anesthesia (Study I, Xylocaine, $10\,\mathrm{mg\,ml^{-1}}$ with adrenalin $5\,\mu\mathrm{g\,ml^{-1}}$, AstraZeneca, Oslo, Norway; Study II, Lidocaine Mylan, $10\,\mathrm{mg\,ml^{-1}}$, Mylan Ireland Ltd, Ireland) with a fine needle (12-14 gauge; Universal-plus, Medax, Italy) operated with a spring-loaded instrument (Bard Magnum, Bard Norway AS, Norway). Sampling was performed as previously described [255], with modifications. Anesthesia was injected in the subcutaneous tissue with care taken not to inject anesthesia into the muscle itself. Following pilot experiments we decided not to use an insertion cannula as described in [255] as the biopsy needle itself could be used to puncture the skin and muscle fascia. This also resulted in less discomfort. Several passes through the same skin puncture was made to obtain sufficient material for downstream analyses. A smaller needle (14 vs. 12 gauge) was used to further minimize discomfort in Study II where more biopsies were sampled over a shorter time span, with exception from when material was used for immunohistochemistry. The first biopsy was sampled at one third of the distance between the patella to the *anterior superior iliac spinae* with subsequent biopsies sampled $\sim$2 cm proximal to previous samples. In Study II, samples obtained more than one week apart were sampled with closer proximity and distally from previous samples but never at previous sampling sites.

The micro biopsy technique produces smaller samples compared to other biopsy techniques [256], and thus requires several passes to produce sufficient material for multiple downstream experiments. However, reports confirms that the micro biopsy technique is comparable to the traditionally used Bergström technique in several measures of muscle characteristics at the same time as being well tolerated

[255,257]. Any reported differences in fiber type distributions between sampling techniques have been suggested relating to differences in sampling depth [257,258].

For determination of fiber type distributions, a threshold of 200-300 fibers has been suggested as a suitable sample size per specimen as more fibers does not reduce the variation between dupliacte samples [259]. In Study I, one or several pieces of muscle (total weight ∼15 mg) were chosen per sampling for analysis of fiber type distributions (described in detail below). The total number of fibers were counted from these specimens (Figure ref fig). Using an average of fibers from the first sampling time point the between leg coefficient of variation was determined to 14% for Type I fibers and 11.3 for type II fibers. The between leg variation in Type I fibers is similar to what has been previously reported...

Total RNA extraction

Total-RNA was extracted from frozen muscle samples with weights measured at collection using a protocol modified from [260] using Trizol reagent (Life Technologies). Muscle tissue was homogenized in 300 ul Trizol with mechanical disruption achieved by Zirconium Oxide Beads (0.5 mm, Next Advance, Inc., New York, USA) and a bead mill (Bullet blender, Next Advance). External, non-mammalian RNA (Lambda PolyA External Standard Kit, Takara Bio Europe, Saint-Germain-en-Laye, France) was added with the initial volume of Trizol to enable per-weight normalization in subsequent analyses. After homogenization, additional Trizol was added to a total volume of 1 ml. Phase separation was achieved by centrifugation after addition of chloroform (200 ul). The upper phase (400 ul in Study I; 450 ul in Study II) was transferred to a fresh tube and RNA was precipitated using isopropanol (500 ul). After incubation (10 min, room-temperature) and centrifugation (12000 g, 10 min at 4°C) the resulting RNA pellet was washed three times in chilled 75% ethanol.

As previously mentioned, to minimize discomfort with a larger number of biopsies sampled over a short time, a smaller needle was used for most biopsies in Study II. This generally led to a less tissue used for RNA extractions (Figure XX).

Protein extraction immunoblotting

Aliquots of muscle-tissue (approximately 25 mg wet weight) were homogenised using a plastic pestle in ice-cold lysis buffer (2 mM HEPES pH 7.4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 1% Triton X-100) spiked with protease and phosphatase inhibitors (Halt, Thermo Fischer Scientific, Life Technologies AS, Oslo Norway),

incubated at 4° for 1 hr and centrifuged for 10 min at 10 000 g and 4°C, after which the supernatants were collected. Total protein concentrations were determined on a 1:10 dilution (Pierce Detergent Compatible Bradford Assay Reagent, Thermo Fischer Scientific). The remaining supernatant was diluted to 1.5 $\mu g \times \mu l^{-1}$ total protein in lysis buffer and 4X Laemmli sample buffer (Bio-Rad Laboratories AB, Oslo Norway) containing 2-Mercaptoethanol. Samples were heated to 95°C for 5 min and stored at -20°C until further processing. During analyses, protein samples (20 μg of total protein) were separated at 300 V for 30 min using 4-20% gels (Criterion TGX, Bio-Rad), followed by wet transfer to PVDF membranes (0.2 μm Immun-Blot, Bio-Rad) at 300 mA for 3 h. Gel electrophoresis and protein transfer were performed at 4°C. Membranes were then stained using a reversible total protein stain (Pierce Reversible Protein Stain, ThermoFischer Scientific) to ensure appropriate protein transfer. Primary antibodies were purchased from Cell Signaling Technology (Leiden, The Netherlands): mTOR (mTOR$^{\text{Ser2448}}$: #5536; pan: #4517), S6 kinase 1 (p85 S6K1$^{\text{Thr412}}$: #9206; p70 S6K1$^{\text{Thr389}}$: #9234; pan: #2708), ribosomal protein S6 (rpS6$^{\text{Ser235/236}}$: #4858; pan: #2317). Membranes were blocked for 2 h in tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl) containing 3% bovine serum albumin and 0.1% Tween-20, followed by over-night incubation with primary antibodies targeting either the phosphorylated or non-phosphorylated epitope diluted in blocking buffer, followed by 2 h incubation with secondary horseradish peroxidase-conjugated antibodies diluted in TBS containing 0.1% Tween-20 and 5% skimmed milk. Membranes were washed in TBS containing 0.1% Tween-20 for 6 × 5 min after incubation with primary antibody, and for 8 × 5 min after incubation with secondary antibodies. For rpS6 and mTOR antibodies, following chemiluminescent detection (SuperSignal™ West Femto Maximum Sensitivity Substrate, ThermoFischer Scientific), membranes were incubated with hydrogen peroxide (15 min, 37°C) to inactivate the horseradish peroxidase (HRP), as described by Sennepin *et al.* [261], followed by over-night incubation with primary or secondary antibodies as described above. If the phosphorylated epitope was targeted during the first incubation, antibodies for the non-phosphorylated epitope were used in the second and vice versa. HRP inactivation did not affect the phosphospecific to non-phosphorylated signal ratios. Importantly, as this technique did not involve removing the first primary antibody, antibodies from different hosts (mouse or rabbit) were used for phosphorylated and non-phosphorylated epitopes respectively. As the antibody targeting p70 S6K1$^{\text{Thr389}}$ had the same host as the pan-antibody, total-protein was used to normalise chemiluminescent signals. All incubation and washing steps were performed at 4°C using an automated membrane

processor (BlotCycler, Precision Biosystems, Mansfield, MA, USA), except for p70 S6K1 experiments, which were performed by hand at room temperature with incubations at 4°C. For mTOR and rpS6, total-protein and chemiluminescence quantification was calculated as the mean value of two separate experiments. S6K1 was quantified once for each phospho-specific antibody. Total-protein content was quantified using ImageJ [262], and was defined as the mean grey value of the whole well with between-well values subtracted as background. Chemiluminescence signals were quantified using Image Studio Lite (LI-COR Biotechnology, Lincoln, Nebraska USA).

Protein was extracted from Trizol preparations according to manufacturers instructions and [263] with modifications. The remaining aqueous phase was removed and DNA was precipitated by the addition of 300 ul of absolute ethanol followed by gentle centrifugation (2000 g, 5 min at room temperature). An aliquot of the phenol-ethanol phase, corresponding to ~1.75 mg of tissue, was transferred to to a fresh tube. After addition of at least two volumes of isopropanol and incubation (10 min at room temperature), samples were centrifuged (7500 g, 10 min 4°C) and a pellet formed. The pellet was washed three times in 95% ethanol with each wash separated by centrifugation (5000g, 5 min at room temperature). After the last wash all liquid was removed and 45 ul of Kopec buffer [263] was added (5% SDS, 10 mM Tris, 140 mM NaCl and 20 mM EDTA, pH 8; containing protease and phosphatase inhibitors). Pellets were incubated at 50°C for three hours after which the majority of samples were dissolved. Any undissolved material was sedimented by centrifugation (10000 g, 10 min at room temperature). Protein concentrations were measured (Pierce Detergent Compatible Bradford Assay, ThermoFisher Scientific). Sample were normalized in 4X Laemmli buffer (Bio-Rad Norway AS, Oslo, Norway), boiled (95°C, 5 min) and stored at -20°C before later use.

4.6 RNA analysis

Complementary DNA (cDNA) was synthesized in technical duplicates from 500 ng of total RNA using random hexemer and anchored Oligo-dT primers (Thermo Fisher Scientific) together with Superscript IV (Thermo Fisher Scientific) according to manufacturer's instruction. qPCR reactions were performed with diluted cDNA (2 ul, 1:25 dilution), a SYBR-green based commercial master mix (PowerUp™ SYBR™ Green Master Mix, Thermo Fisher) and, target-specific primers (500 nM) in 10ul reaction volumes using a real-time detection system (QuantStudio 5 Real-Time PCR System, Thermo Fisher Scientific). Fast cycling was used (1 sec

denaturing, 30 sec annealing) after UNG (2 min, 50°C) and polymerase (2 min, 95°C) activation. Melt curves were collected from all reactions to confirm single product amplification. Primers were further evaluated by agarose gel electrophoresis which confirmed primer sizes and non-template control experiments confirming no amplification without template. Primer sequences and their respective average performances are shown in Table 2.

Approximately 25 mg of wet muscle-tissue was homogenised in a total volume of 1 ml of TRIzol reagent (Invitrogen, Life technologies AS, Oslo, Norway) using 0.5 mm RNase-free Zirconium Oxide beads and a bead homogeniser (Bullet Blender, Next Advanced, Averill Park, NY, USA) according to the manufacturer's instructions. In order to enable analysis of target gene-expression per-unit tissue weight, an exogenous RNA control (λ polyA External Standard Kit, Takara Bio Inc, Shiga, Japan) was added at a fixed amount ($0.04\ ng \times ml^{-1}$ of Trizol reagent) per extraction prior to homogenisation, as previously described [264, 265]. Following phase-separation, 400 μl of the upper phase was transferred to a fresh tube and RNA was precipitated using isopropanol. The resulting RNA pellet was washed three times with 70% EtOH and finally eluted in TE buffer. RNA quantity and purity was evaluated using a spectrophotometer, all samples had a 260/280 nm ratio > 1.95. RNA was stored at -80°C until further processing. In the analysis of total RNA content per-unit tissue weight, one sample was excluded prior to analysis due to negative deviation from the expected value based on the relationship between sample weight and RNA content suggesting sample loss in washing steps. RNA integrity was assessed by capillary electrophoresis (Experion Automated Electrophoresis Station using RNA StdSens Assay, Bio-Rad) with average integrity scores (RQI) Five-hundred nanograms of RNA were reverse transcribed using anchored Oligo-dT, random hexamer primers (Thermo Scientific) and SuperScript IV Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. All samples were reverse transcribed in duplicates and diluted 1:50 prior to real-time polymerase chain reaction (qPCR). qPCR reactions were run on a fast-cycling real-time detection system (Applied Biosystems 7500 fast Real-Time PCR Systems, Life technologies AS), with a total volume of 10 μl, containing 2 μl of cDNA, specific primers (0.5 μM final concentration) and a commercial master mix (2X SYBR Select Master Mix, Applied Biosystems, Life technologies AS). qPCR reactions consisted of 40 cycles (three seconds 95°C denaturing and 30 seconds 60°C annealing). Melt-curve analyses were performed for all reactions to verify single-product amplification. Gene-specific primers were designed for all targets using Primer-BLAST [266] and Primer3Plus [267] and ordered from

Thermo Scientific, except for the external RNA control, for which primers were supplied with the kit. Raw fluorescence data was exported from the platform specific software and amplification curves were modelled with a best-fit sigmoidal model using the qpcR-package [268] written for R [**R-base?**]. Threshold cycles (Ct) were estimated from the models by the second-derivate maximum method with technical duplicates modelled independently. Amplification efficiencies were estimated for every reaction [as described by 269,implemented in 268]. For every primer pair, mean amplification efficiencies (E) were utilised to transform data to the linear scale using E^{-Ct}. Primer sequences and primer characteristics (i.e. average primer efficiencies and Ct values) are presented in Table 2. Gene expression data was log-transformed prior to statistical analysis. As Ct-values, but not efficiencies are related to RNA integrity [270], RQI scores were used in the statistical treatment of qPCR data to control for potential degradation effects on a by target basis (see below).

4.6.1 Hormonal measurements

Hormone analyses were performed on blood samples collected at five time points: alongside muscle biopsies (Figure 1, four sampling events) and 10 minutes after completion of the fifth training session. Samples were drawn from the antecubital vein into serum-separating tubes and kept at room temperature for 30 min before centrifugation (1500 g, 10 min). Serum was immediately aliquoted and stored at -80°C until further processing. Serum concentrations of total testosterone, cortisol, growth hormone and insulin-like growth-factor 1 (IGF-1) were measured on an Immulite 1000 analyzer, using kits from the Immulite Immunoassay System menu (Siemens Medical Solutions Diagnostics, NY, USA), performed according to manufacturer's protocols. Serum Vitamin D (S-25-OH-D) levels were measured in samples collected before and after the intervention using a electrochemiluminescence immunoassay (Roche Cobas Vitamin D total assay, Roche Diagnostics GmbH., Mannheim, Germany) using automated instrumentation (Roche Cobas 6000's module e601, Roche Diagnostics GmbH., Mannheim, Germany).

4.7 Statistics and data analysis

In Study I, an *a priori* sample-size calculations ($\beta = 20\%$, $\alpha = 5\%$) indicated that 40 participants was sufficient to detect $\sim 3\%$ and 5%-point differences between volume conditions in primary outcomes, training induced changes in muscle cross-sectional

area and maximal voluntary strength respectively. Sample-size calculations were based on assumed differences between volume condition corresponding to effect sizes of 0.47-0.51, as estimated from previous studies [145,146].

In Study II, an initial sample size calculation was made based on a best case scenario. Data from Brook et al. [271] indicated that a within-participant differences in RNA per tissue weight of 15% could be detected with seven participants in the experimental group (e.g. 2.79 (0.65) to 3.21 (0.74) $\mu g \mu l^{-1}$, $\alpha < 0.05$, $\beta < 0.2$, effect size=1.2). The control group was included in the study as a negative control primarily for the sake of systems validation of experimental procedures regarding measures of e.g. ribosomal biogenesis. With a balanced design, accounting for drop-outs eight participants were required in each group. After a preliminary analysis of the experiment (experimental group $n = 7$, control group $n = 7$)[272], additional participants (experimental group $n = 4$, control group $n = 1$) were recruited to the study to increase the precision of estimates, primarily in analyses within the experimental group.

TO DO:

- For methods discussion, compare product length, efficiencies and ct values in relation to RQI-values. See Fleige 2006 for reference.

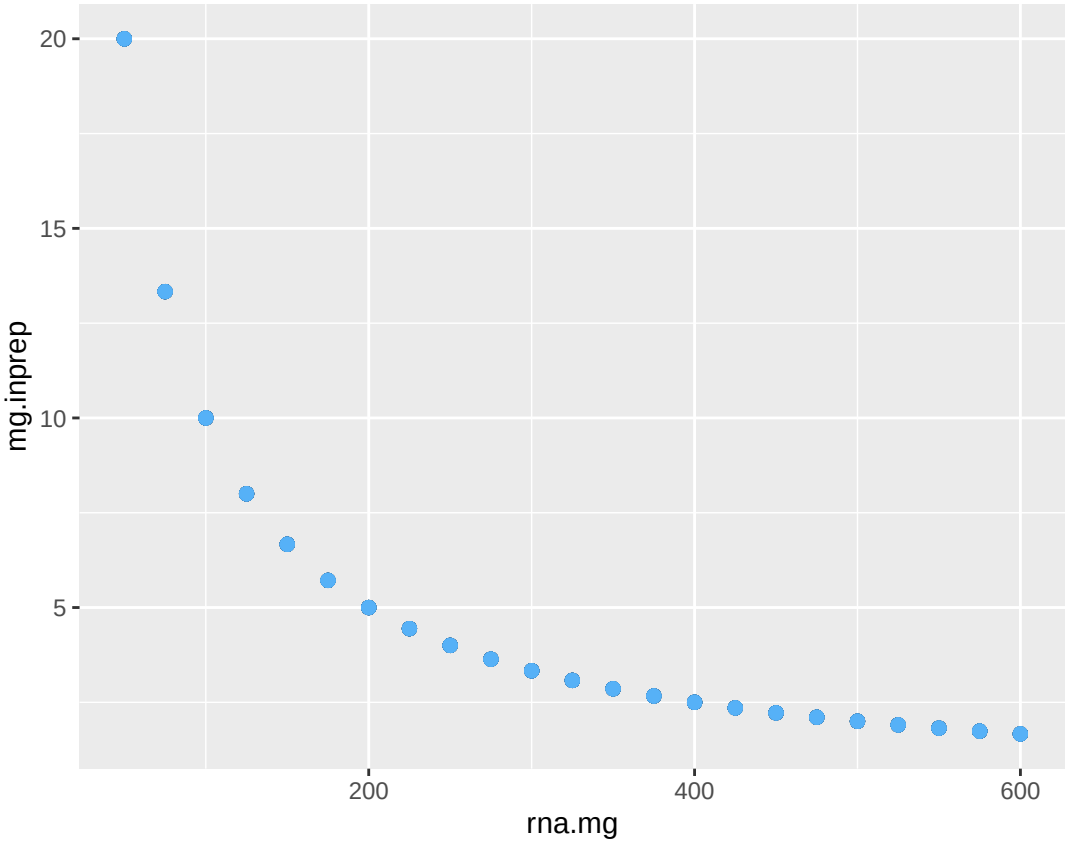
4.7.1 Normalization

- An external reference gene was added at a constant amount in Trizol preps
- A normalization factor was used to express relative target gene abundance per-weight tissue.
- In qPCR the linearised expression (effectivety ^cq) was used to express the fraction of external reference per total RNA.
- In RNA-seq the external reference gene was sequenced and counts were used to express external RNA as a fraction of total RNA.
- In both cases the normalization factor was calculated as mw * counts.

A simulation to see that this is equivalent to tissue used in prep when no measurement errors exists.

```
# A tibble: 300 x 7
      mg rna.mg   ext tot.rna  ext.frac mg.inprep        nf
   <dbl>  <dbl> <dbl>   <dbl>     <dbl>     <dbl>     <dbl>
 1     5    250  0.04    1250 0.0000320      4.00 0.000160
 2     5    275  0.04    1375 0.0000291      3.64 0.000145
```

```
 3     5   300  0.04   1500 0.0000267      3.33 0.000133
 4     5   325  0.04   1625 0.0000246      3.08 0.000123
 5     5   350  0.04   1750 0.0000229      2.86 0.000114
 6     5   375  0.04   1875 0.0000213      2.67 0.000107
 7     5   400  0.04   2000 0.0000200      2.50 0.000100
 8     5   425  0.04   2125 0.0000188      2.35 0.0000941
 9     5   450  0.04   2250 0.0000178      2.22 0.0000889
10     5   475  0.04   2375 0.0000168      2.11 0.0000842
# ... with 290 more rows
```



4.8 Meta-analysis of within-session training volume

4.8.1 Literature search, inclusion criteria and coding of studies

A first set of studies were coded based previously published meta-analyses [24,25]. For more recent studies, PubMed, Google Scholar and SportDiscuss searches were made with search terms being "training volume," "resistance training," "strength training," "set," "muscle strength," "muscle hypertrophy" used in different combinations. Studies examining the effect of within-session training volume on muscle strength and mass, with all other training variables kept constant between study groups were considered for inclusion in the meta-analysis. Studies were further assessed for inclusion based on criteria being; (i) participants described as healthy without medications affecting muscle metabolism, (ii) interventions lasting at least 6 weeks and (iii) RT performed without additional stimuli (e.g. blood flow restriction) at intensities above 65% of 1RM or 20RM.

All available outcome measures of muscle mass and strength gains in response to RT were extracted from each study with exception of outcomes reported both as summaries and individual measures (e.g. muscle thickness reported as individual muscles and summarized for the whole muscle group). In such cases the summary was used as outcome. Weekly training volume was calculated as product of weekly sessions, number of sets and exercises for each muscle group assessed for muscle hypertrophy or strength gains. An intervention average of weekly sessions was used when the number of sessions per week differed over the course of the intervention. An exercise was assumed to influence an outcome when it targeted prime movers also assessed for strength or muscle hypertrophy measures. Participant characteristics were coded based on sex (male, female or mixed when a study failed to discern between male and females), age (young, middle-aged, old or mixed), body-mass index (BMI, calculated from average body mass and height when BMI values were not available), training status (trained, $>$ 1 session per week during the last 6 months leading up to the intervention; untrained $<$ 1 session). Study groups were considered independent also in studies utilizing within-participant models.

4.8.2 Calculations of effect sizes and statistical analysis

Group-wise effect sizes were calculated for each outcome measure based on the within-group change score pre- to post-training divided by the pre-training standard deviation (SD). Pre-training SD's were calculated as a pooled SD within outcome and study. Variances of the effect size were calculated using an average effect size across all outcomes within muscle strength or mass, and correlations specific to each measurement type (isokinetic-, isometric- or repetition maximum strength tests;

muscle thickness, magnetic resonance imaging, dual energy X-ray absorptiometry) estimated from previous studies.
A correction factor () was applied to both effect sizes and their variances.

Mixed-effects meta-regression models were used to model the effect of weekly number of sets on RT-induced muscle mass and strength gains. Models were fitted in a Bayesian framework using the brms-package [273].

5. Results and Discussion

5.1 Effects of different training volume on changes in muscle size and function

In Study I, the average increases in muscle strength and mass in each volume condition corresponded to what could be expected based on previous research in young, healthy participants (Table 5.1) [274, 14], indicating the general efficacy of the training program.

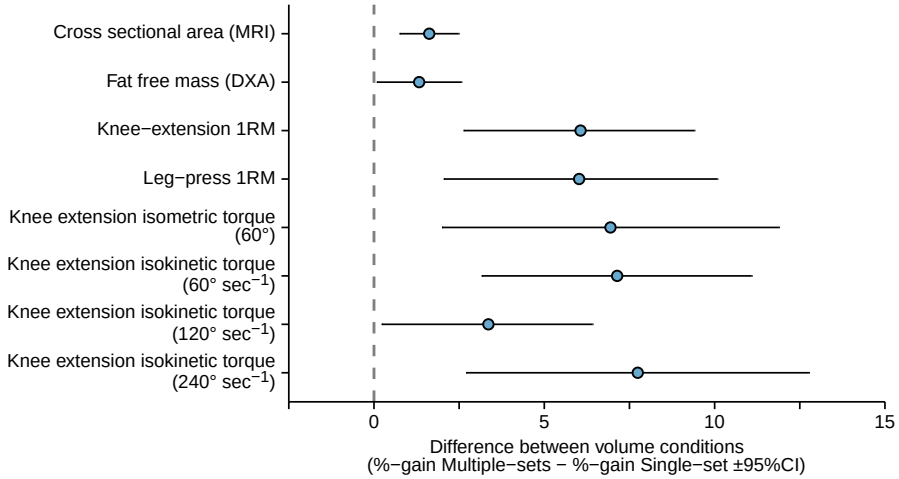


Figure 5.1: Differences in training induced relative changes in muscle mass and strength measures. Estimates are derived from ANCOVA models controling for baseline values.

The multiple-sets (moderate-volume) condition consistently showed favorable adaptations when compared to the single-set (low-volume) condition in measures of muscle hypertrophy and strength gains (Figure 5.1).

In an attempt to explain differences in training outcomes between volume-conditions, selected molecular markers with known influence on adaptations to resistance training were investigated for volume-dependency. First, acute activation

Table 5.1: Training induced changes in muscle CSA and average strength in Study I

Sex	Condition	Mean (SD)	Reference
CSA %-change			
Female	LOW	3.05 (3.61)	
	MOD	5.02 (4.04)	
Male	LOW	3.83 (3.50)	
	MOD	5.10 (3.71)	
CSA %-change per day			
Female	LOW	0.04 (0.05)	
	MOD	0.07 (0.05)	
Male	LOW	0.05 (0.05)	0.11 [0.04-0.26]a
	MOD	0.07 (0.05)	
CSA %-change per session			
Female	LOW	0.11 (0.13)	
	MOD	0.18 (0.15)	0.08 (0.22)b
Male	LOW	0.14 (0.12)	
	MOD	0.19 (0.13)	0.14 (0.14)b
Average strength %-change			
Female	LOW	21.0 (9.8)	
	MOD	27.8 (14.4)	
Male	LOW	19.2 (12.4)	
	MOD	23.1 (12.0)	
Average strength %-change per session			
Female	LOW	0.77 (0.36)	
	MOD	1.00 (0.49)	0.67 (0.35)b
Male	LOW	0.72 (0.48)	
	MOD	0.87 (0.46)	0.47 (0.22)b

[a] Estimates from Wernbom et al. [274]
[b] Estimates from Ahtiainen et al. [14]

of signaling along the mechanosensitive mTORC1-pathway has previously been shown to correlate with training-induced muscle growth [157, 168, 275], presumably trough increased translation indicated by greater ribosomal association with mRNA [157]. A commonly used readout of mTORC1-signaling is the phosphorylation of S6-kinase (S6K1) at Thr^{389}/Thr^{412} which in turn precedes phosphorylation of ribosomal protein S6 (rpS6, see Figure 5.2a). In the present study, exercise-induced $S6K1^{Thr^{389}/Thr^{412}}$ phosphorylation was indeed shown to be volume dependent along with phosphorylation of $rpS6^{Ser^{235/236}}$ and $mTOR^{Ser^{2448}}$ (Figure 5.2 b). It is recognized that phosphorylation of mTOR itself at Ser^{2448} primarily should be regarded as indicative for negative feedback as this site is phosphorylated due to S6K1 activity [276] (Figure 5.2 a). It is also recognized that the phosphorylation status of rpS6 at $Ser^{235/236}$ is not solely due to mTORC1 signalling as both mTORC1 and extracellular signal-regulated kinases (ERK)-signalling converges here [226]. Together these observations indicates larger perturbations along the mTORC1 signaling axis which confirms previous observations showing exercise-volume dependency of mTORC1 related signaling
[20, 21, 22]. Interestingly, albeit with limited resolution, Terzis et al., did not find any clear volume dependency in exercise induced activation of either ERK 1/2 nor p38 [168] suggesting that the relative contribution of different pathways to activation of downstream effectors may differ depending on mechanical stimuli. It should however be noted that the present, and previous studies are limited in their temopral resolution and different patterns over time in different volume conditions cannot be ruled out.

The importance of mTORC1 signalling in protein synthesis in the acute phase after resistance exercise is well established. In humans, administration of rapamycin, a selective inhibitor of mTORC1, prior to resistance exercise leads to delayed or blunted activation of mTORC1 effectors such as $S6K1^{Thr^{389}}$, $S6K1^{Thr^{421}/Ser^{424}}$, $rpS6^{Ser^{235/236}}$ and $rpS6^{Ser^{240/244}}$ as well as unchanged levels of phopshorylation of 4E-BP1 and eEF2 concomitantly with abolished exercise-induced increase in protein synthesis [169]. Volume dependent increase in mTORC1 signalling also coincide with larger protein synthesis rates [20]. As such, volume dependence of mTORC1-related signalling suggests that higher within-session volume can be regarded as leading to an increased potential for protein synthesis in the acute-phase after exercise. However, an increased signalling through this pathway could also be interpreted as indicative for increased ribosomal biogenesis.

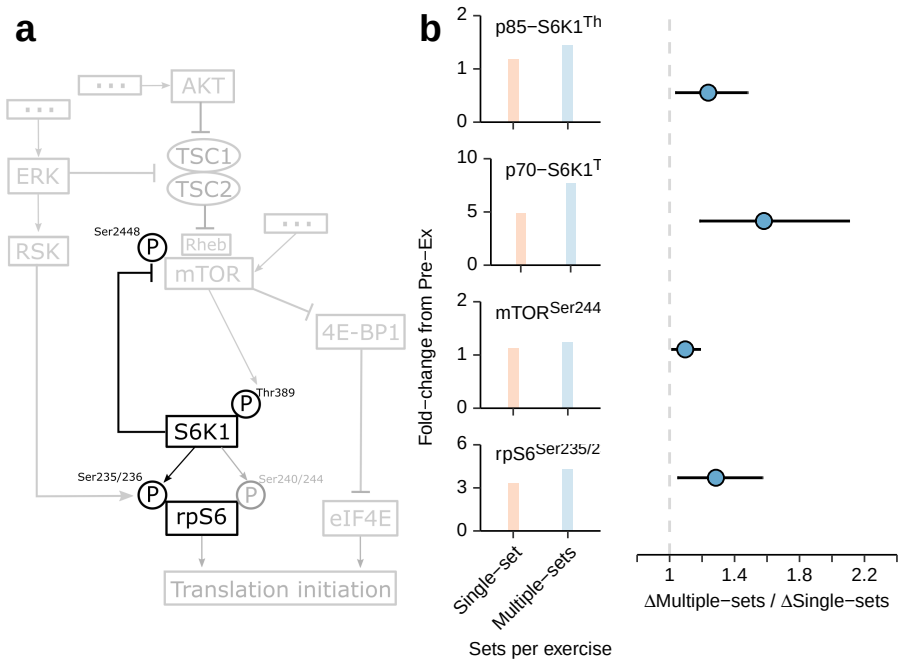


Figure 5.2: Measured phosphorylation sites in context (a) and differences between volume conditions in phosphorylation status of S6K1 at Thr ^{389} (p70) Thr ^{412} (p85), rpS6 at Ser $^{235\ 236}$ and mTOR at Ser ^{2448} induced by acute exercise and expressed as fold-changes (b). Estimates are derived from ANCOVA models controling for baseline values. Values in b are point estimates with 95% confidence intervals.

Indeed, mTORC1 affects ribosomal biogenesis through multiple mechanisms including activation of transcriptional programs related to ribosomal biogenesis (mediated through S6K1) [187], selective translation of specific mRNA related to ribosomal biogenesis [189] which in turn stimulates to increased ribosomal RNA transcription, mediated by cyclin D1 abundance and UBF availability [210].

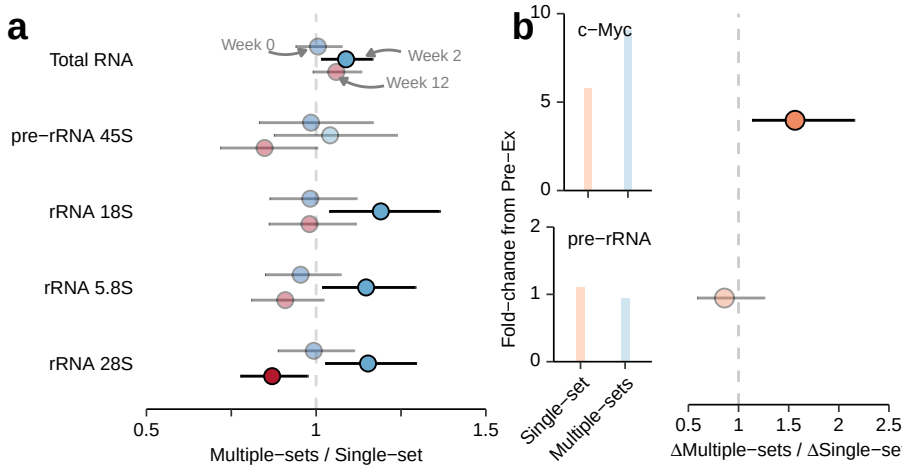


Figure 5.3: Differences between volume conditions in total RNA and ribosomal RNA-species (pre-rRNA 45S, rRNA 18S, rRNA 5.8S and rRNA 28S) measured at rest during the course of Study I (a). Acute changes in abundance of c-Myc mRNA and pre-rRNA 45S in response to acute exercise in Week 2 and differences between volume conditions (b). Errorbars represents 95% confidence intervals, transparent points and errorbars signifies that the confidence interval contain 1.

As ribosomal RNA is the most abundant constituent of muscle RNA it provides an estimate of muscle ribosomal abundance when expressed per unit tissue weight [162, 164]. From baseline to prior to the fifth training session (Week 2), total RNA per mg tissue increased by $\sim$ 19 and 34% in the single-set and multiple-sets condition, respectively. Total RNA levels were still elevated above baseline after the intervention (Week 12, single-set $\sim$ 13; multiple-sets $\sim$ 20%). Similar patterns were seen in target analysis of ribosomal RNA using qPCR (single-set increase from baseline to Week 2 $\sim$ 8-44% and Week 12 $\sim$ 14-36%; multiple-sets $\sim$ 31-57% and Week 12 $\sim$ 14-23%). Comparing volume conditions revealed higher levels of total RNA and mature ribosomal RNA species in the multiple-sets condition at Week 2 (Figure 5.3a). At Week 12 differences between volume conditions were in total RNA less clear and ribosomal RNA 28S showing higher levels in the single-set leg (Figure 5.3a).

Together with indications of greater mTORC1 activation, analysis of c-Myc mRNA abundance in response to the fifth training session (Week 2) also showed volume-dependent regulation with exercise-induced increases being ~ 1.5-fold in response to the multiple-sets compared to the single-set condition (Figure 5.3b). c-Myc represents a rapamycin-insesitive signalling pathway known to also stimulating ribosomal biogenesis [] []

Table 5.2: Influence of RNA abundance on training-induced muscle growth measured with MRI

						Standardized coefficients
	Estimate	SE	df	*t*-value	95% CI	Estimate [95% CI]
(Intercept)	-9.56	3.53	32	-2.71	[-16.75, -2.38]	4.21 [3.02, 5.39]
RNA abundance (ng mg^{-1})						
Week 0	0.00	0.01	29	0.15	[-0.016, 0.02]	0.05 [-0.62, 0.72]
Week 2	0.02	0.01	29	4.26	[0.012, 0.04]	1.66 [0.86, 2.45]
Week 12	0.01	0.01	29	1.92	[-0.001, 0.02]	0.75 [-0.05, 1.54]

[a] Standardized coefficients scaled by its SD

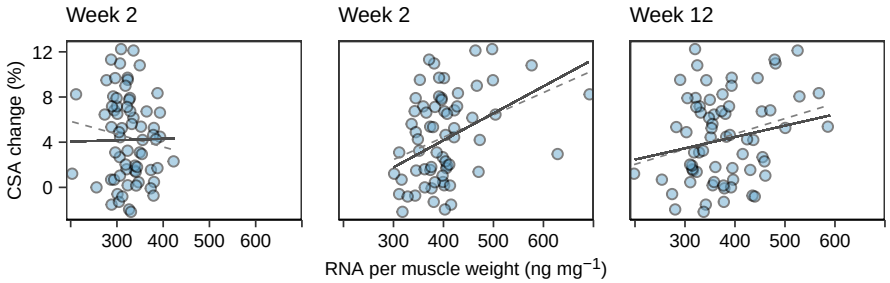


Figure 5.4: Relationship between total RNA and training induced change in thigh cross sectional area (CSA) as measured by MRI. Dashed line represent 'naive' relationship, solid line represent adjusted reliationship as seen in
reftable:rna-csa-tab.

Additionally, results from genetically modified mice lacking the mTORC1 effector S6K1 confirms its specific importance for maintenance of skeletal muscle size as these mice [184]. Particularly S6K1

mTORC controls protein synthesis trough multiple mechanisms 4E-BP1 and S6K1-rpS6

mTORC1 enhance translation of specific mRNA, including ribosoma biogenesis genes

[186]

[277]

Given these limitations in using mTORC-signalling as markers of muscle hypertrophy, it is not surprising that previous studies are ambiguous in their associative approach between acute mTORC1-related phosphorylation and hypertrophy in humans. Some studies find a strong correlation

[146, 278].

[167]; [168];

This seems somewhat counterintuitive, as this pathway is a known regulator of translation initiation and elongation, as well as of ribosomal biogenesis [210, 196, 187, 194]

Indeed, in the present study we observed volume-dependence of mTOR phosphorylation at Ser2448, which could be a sign of negative feedback from mTORC1-based activation of S6K1 [192]. [24]; [23]; [25]]. Furthermore, when a combining data from more recent studies indicates that a higher training volume is generally associated with increased muscle hypertrophy and strength gains (Figure 5.1 and 5.1.

In Study II, training efficacy was assessed by comparing outcomes to a non-training control group. The training group displayed increases compared to the control group for both strength muscle thickness measures.

5.2 Acute effects of diffrent training volume on determinants of muscle protein synthesis

6. General Discussion

Conclusion

If we don't want Conclusion to have a chapter number next to it, we can add the `{-}` attribute.

More info

And here's some other random info: the first paragraph after a chapter title or section head *shouldn't be* indented, because indents are to tell the reader that you're starting a new paragraph. Since that's obvious after a chapter or section title, proper typesetting doesn't add an indent there.

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