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Effects of immobilization by 60 days of experimental bed rest on endomysium of the soleus muscle in humans

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Contents

Abbreviations

AG ..... *artificial gravity*
AGE ..... *advanced glycation end product*
DXA ..... *dual energy X-ray absorptiometry*
ECM ..... *extracellular matrix*
ELISA ..... *enzyme-linked immunosorbent assay*
HDBR ..... *head-down tilt bed rest*
HPLC ..... *high performance liquid chromatography*
IMCT ..... *intramuscular connective tissue*
LBNP ..... *lower body negative pressure*
MRI ..... *magnetic resonance imaging*
MVC ..... *maximal voluntary contraction*
SANS ..... *spaceflight associated neuro-ocular syndrome*
SMS ..... *space motion sickness*

1. Summary

Movements result from contracting skeletal muscles. Muscles adapt to changing loads through hypertrophy (enlargement of muscle mass due to increased load) and atrophy (loss of muscle mass due to decreased load). Muscles are known to atrophy during immobilization and disuse. The two primary components of muscle are the muscle fibers (muscle cells) and intramuscular connective tissue. Intramuscular connective tissue can be categorized into endomysium (the smallest component which engulfs each individual muscle fiber), perimysium (which engulfs muscle bundles), and epimysium (which engulfs the muscle as a whole). It has been suggested that endomysium plays a key role for intramuscular force transmission. Animal data suggests that long-term immobilization results in increased endomysium content in the muscle while the muscle fibers themselves atrophy. This result has to date not been demonstrated in humans. Our objective was therefore to examine the endomysium content of the human soleus muscle during 60 days of bed rest.

Bed rest is an established model to investigate changes occurring in microgravity conditions. For muscle, these changes include, muscle fiber atrophy and a loss of muscle strength. A 6° head down tilt is commonly applied to simulate the altered fluid distribution which occurs in microgravity. The 60-day 6° head-down tilt AGBRESA bed rest study, on which this work is based, sought to examine short duration continuous and intermittent centrifugation, i.e., artificial gravity, as a countermeasure to the adverse effects of microgravity on the human body. A short-arm centrifuge at the :envihab in Cologne, Germany, was used to simulate gravity by pulling blood towards the lower extremities. Muscle biopsies of the soleus muscle, a muscle which bears significant loads during daily tasks such as walking and standing, were extracted from 21 healthy volunteers (14 men and 7 women) at three timepoints: at baseline before bed rest (BDC), and during day 6 and day 55 (HDT6 and HDT55) of the head-down tilt bed rest. The biopsies were frozen, sectioned, and stained for laminin γ-1, a protein of the muscle basement membrane, which surrounds each cell. Laminin γ-1 can be used to determine the intramuscular connective tissue area located in between the individual fibers. Two parameters were calculated: the endomysium-to-fiber-area ratio (EFAr, in %) and the endomysium-to-fiber number ratio (EFNr). EFAr can show an increase in endomysium while EFNr can differentiate between a relative increase, attributed to muscle fiber atrophy, and an absolute endomysium increase.

Muscle fibers atrophied by 16.6 % (SD 28.2 %) at HDT55 ($p=0.0031$) during bed rest. EFAr increased on day 55 ($p<0.001$), while EFNr showed no significant effect of bed rest ($p=0.62$). Neither intervention group showed significant effects in response to the use of short-arm centrifugation as a countermeasure to the adverse effects of microgravity on muscle fiber atrophy.

The results of an EFAr increase and EFNr stability are explainable by immobilization-induced muscle fiber atrophy. Assuming that the number of fibers remains constant during long-term immobilization, these results suggest an unchanged amount of endomysial connective tissue content coupled with an increase in the relative amount of connective tissue per muscle fiber. This change is likely to affect muscle stiffness and thus muscle function.

2. Zusammenfassung

Bewegungen entstehen durch sich kontrahierende Skelettmuskeln. Muskeln passen sich veränderten Belastungen durch Hypertrophie (Vergrößerung von Muskelmasse aufgrund erhöhter Belastung) und Atrophie (Verlust von Muskelmasse aufgrund verringerter Belastung) an. Es ist bekannt, dass Muskeln bei Immobilisierung und Entlastung atrophieren. Die zwei Hauptkomponenten des Muskels sind die Muskelfasern (Muskelzellen) und intramuskuläres Bindegewebe. Intramuskuläres Bindegewebe kann in Endomysium (den kleinsten Bestandteil, der jede einzelne Muskelfaser umgibt), Perimysium (das Muskelbündel umgibt) und Epimysium (das den Muskel als Ganzes umgibt) eingeteilt werden. Es wird angenommen, dass Endomysium für die intramuskuläre Kraftübertragung eine wichtige Rolle spielt. Daten aus Tierstudien weisen darauf hin, dass langfristige Immobilisierung zu erhöhtem Endomysiumgehalt im Muskel führt, während die Muskelfasern selbst atrophieren. Dieses Ergebnis wurde bisher nicht an Menschen nachgewiesen. Unser Ziel war es daher, den Endomysiumgehalt im menschlichen Soleus-Muskel während 60 Tagen Bettruhe zu untersuchen.

Bettruhe ist ein bewährtes Modell um in Mikrogravitation auftretende Veränderungen zu untersuchen. Für den Muskel beinhaltet dies unter anderem Muskelfaseratrophie und den Verlust von Muskelkraft. Um die geänderte Flüssigkeitsverteilung in Mikrogravitation zu simulieren wird häufig eine 6° Kopftieflage verwendet. Die dieser Arbeit zugrunde liegende AGBRESA 60-Tage 6° Kopftieflage Bettruhestudie beabsichtigte kurzzeitige kontinuierliche und intermittierende Zentrifugation, d.h. künstliche Schwerkraft, als Gegenmaßnahme zu den negativen Effekten der Mikrogravitation auf den menschlichen Körper zu untersuchen. Eine Kurzarmzentrifuge im :envihab in Köln, Deutschland, wurde benutzt um Schwerkraft zu simulieren, indem Blut in die unteren Extremitäten gezogen wird. Bei 21 gesunden Probanden (14 Männer und 7 Frauen) wurden aus dem Soleus-Muskel, einem Muskel, der im Alltag durch Laufen und Stehen erhebliche Lasten trägt, Muskelbiopsien zu drei Zeitpunkten entnommen: bei Baseline vor Bettruhe (BDC) sowie an Tag 6 und Tag 55 (HDT6 und HDT55) der Bettruhe in Kopftieflage. Die Biopsien wurden eingefroren, geschnitten und für Laminin γ-1 gefärbt, ein Protein der Basalmembran, welche jede Zelle umgibt. Laminin γ-1 kann zur Bestimmung der intramuskulären Bindegewebsfläche, die zwischen den einzelnen Muskelfasern lokalisiert ist, genutzt werden. Es wurden zwei Parameter berechnet: das Endomysium-zu-Faserfläche-Verhältnis (EFAr, in %) und das Endomysium-zu-Faseranzahl-Verhältnis (EFNr). EFAr kann eine Zunahme des Endomysiums anzeigen, während EFNr zwischen einer relativen Zunahme, die der Muskelfaseratrophie zuzurechnen ist, und einer absoluten Endomysiumzunahme unterscheiden kann.

Wie erwartet, atrophierten Muskelfasern während Bettruhe um 16.6 % (SD 28.2 %) an Tag HDT55 ($p=0.0031$). EFAr nahm an Tag 55 zu ($p<0.001$), während Bettruhe keinen

signifikanten Effekt auf EFNr zeigte (p=0.62). Keine der beiden Interventionsgruppen zeigte signifikante Effekte bezüglich der Wirksamkeit der Kurzarmzentrifugation als Gegenmaßnahme zu den negativen Auswirkungen der Mikrogravitation auf Muskelfaseratrophie.

Die Ergebnisse der EFAr-Zunahme und EFNr-Stabilität sind durch immobilisationsinduzierte Muskelfaseratrophie erklärbar. Unter der Annahme, dass die Anzahl an Muskelfasern während langfristiger Immobilisation konstant bleibt, deuten diese Ergebnisse auf einen unveränderten endomysialen intramuskulären Bindegewebsgehalt hin, wobei der relative Bindegewebsanteil pro Muskelfaser zunimmt. Es ist anzunehmen, dass diese Veränderung die Muskelsteifheit und somit die Muskelfunktion beeinflusst.

3. Introduction

3.1. Challenges in Spaceflight

3.1.1. Physiological effects of microgravity

Adaptive processes of the human body have been reported by both astronauts and cosmonauts since spaceflight begun. As reviewed by Hodkinson et al., weightlessness, or microgravity, influences almost every system of the human body, most importantly the musculoskeletal, the cardiovascular and the neurovestibular systems[1].

Weight-bearing muscles atrophy in the absence of gravitational loading, resulting in a loss of strength, most prominently in the lower extremity[1–3]. Bone density is thereby lost and the ligaments of the spine lengthen, causing back pain[4–6]. Fluid shifts occur in a cranial direction, resulting in the recognizable 'puffy face' and 'chicken legs'[7]. The amount of blood plasma decreases, resulting in relative hypovolemia, which in turn affects the orthostatic tolerance upon a return to a gravitational environment[7,8]. Radiation exposure increases as the duration of space flight increases, which is in turn assumed to increase the risk of tumors and degenerative diseases[9]. Neurological system changes in microgravity conditions contribute to the various forms of space motion sickness (SMS) which include nausea, vomiting and disorientation[10]. In addition to SMS, findings of sight degradation summarized under the term 'spaceflight associated neuro-ocular syndrome' (SANS) contribute to highly relevant neurological impairments. Symptoms include optic disc edema, globe flattening, and ischemic spots in the retina called cotton wool spots[11,12]. In addition thereto, the electrolyte balance, the hormonal balance, the proprioceptive system (resulting in neurosensory disturbance post-flight), the immune system, as well as sleep patterns are all disrupted or changed. All gravitational stimuli are missing in microgravity, resulting in a disuse of these respective sensors. [1,13]

No less important than the physical changes are the psychological effects of isolation and confinement over extended periods of time, which lead to potential conflicts with crew members and a reduced work-output.

As set forth above, microgravity influences many fundamental functions of the human body. It is important to not only study these symptoms and systems individually, but to also focus on their respective interactions with each other.[1,14]

3.1.2. Ground-based microgravity analogs – the bed rest model

Since very few human beings, and specifically astronauts and cosmonauts, have been allowed to experience microgravity conditions over an extended time period, a more accessible way is needed to study changes occurring in microgravity conditions. Multiple models have to date

been developed and are applied depending on a study's objective. Although these models cannot precisely simulate all microgravitational changes, they can approximate effects so as to allow a closer examination.

Physical and psychological effects are mostly studied separately. While isolation studies simulate psychological changes, the most commonly applied models to approximate physical effects are bed rest, water immersion, and dry immersion[15,16]. The bed rest model has become the most commonly used due to its ease of implementation[15]. In addition to simulating the effects of microgravity (even if changes occur more slowly than with dry immersion or spaceflight), bed rest studies can also examine the effectiveness of various countermeasures[17].

One widely established analog for microgravity is the bed rest model with a 6° head-down tilt (HDBR) which is in particular used to simulate conditions for the musculoskeletal and cardiovascular systems. It is based on the redistribution of gravitational forces, leading to a cephalad fluid shift (as shown in Figure 1) and the strict limitation of movement, which induces the immobilization of muscles, in particular those in the lower limbs.

To examine skeletal muscle adaptations, the most commonly applied models are experimental bed rest, dry immersion, unilateral limb suspension, and the use of casts and orthoses in humans, as well as hindlimb unloading in rodents. While the use of casts and orthoses and unilateral limb suspension target the analysis of one specific structure, e.g., muscle, experimental bed rest and dry immersion take a more systematic approach and can simultaneously examine multiple systems while also take the interactions of these multiple systems into consideration.

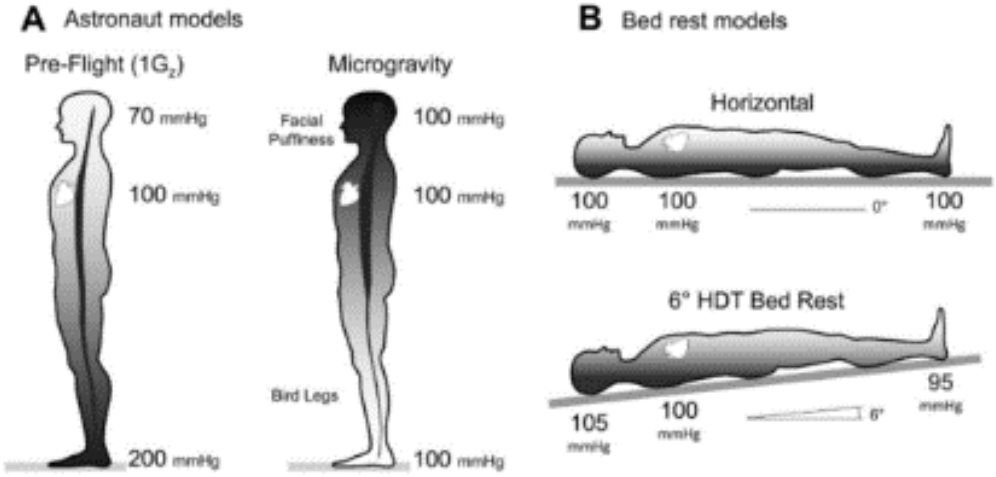

Figure 1 Fluid shift during microgravity, bed rest and 6° head-down tilt bed rest. Gravity forces fluids downwards towards the ground on Earth. This is reversed in microgravity where an "upward" fluid shift results. This "upward" fluid shift can be mimicked more accurately with head-down tilt bed rest than with horizontal bed rest. While the fluid shift in microgravity and 6° head-down tilt is similar, the arterial blood pressures differ.[14]

3.1.3. Countermeasures and artificial gravity

With the objectives of returning to the moon or of venturing to Mars, realizing effective countermeasures becomes essential. The physical and mental well-being of a crew must be ensured to provide for their safety and to allow for the efficient performance of required tasks. Countermeasures for muscle changes gain additional scientific significance since immobilization is not only relevant in spaceflight, but also in clinical settings, such as rehabilitation after surgery or hospitalization and aging.

Multiple types of countermeasures have to date been explored to prevent physical deconditioning. Countermeasures originally focused mostly on exercise protocols to counteract inactivity of the musculoskeletal system. Multiple approaches concerning resistive exercise protocols as well as protocols focused on endurance training exist. The effects of nutrition, lower body negative pressure (LBNP) and its combination with exercise, the combination of vibration and exercise, pharmacological interventions, as well as the use of artificial gravity via a short-arm human centrifuge, have also been examined[17]. These have to date only achieved limited success, are not sufficiently effective, and if so, are often limited to one system.

Artificial gravity (AG) has been postulated as a potential step towards an effective countermeasure response since it stimulates all physiological functions in the human body and is omnipresent on Earth. AG can counteract changes in the cardiovascular system as well as muscle atrophy, bone loss, and neurovestibular deconditioning, and can therefore be regarded as an integrated countermeasure[18]. It has been hypothesized that artificial gravity can be used as a countermeasure to muscular deconditioning since gravity on Earth inhibits muscle atrophy through simple tasks such as walking[2].

3.2. Muscle fibers

Muscle fibers (also called muscle cells or myofibers) are the active contractile elements of a muscle. The contractile properties stem from myofilaments, mostly actin and myosin, which form sarcomeres. These basic proteins are responsible for force generation through myofilament sliding and make up the characteristic striation of skeletal muscle seen in light microscopy. Additional proteins (i.e., troponin and tropomyosin) are involved in contraction which have regulatory and anchoring functions[19].

The thin actin filaments and multiple other proteins, including titin and nebulin, are anchored in the Z-disk which marks the border of the sarcomere axially, so that one sarcomere is arranged between two respective Z-disks[20]. Alpha-actinin is thereby one of the main anchoring components[20,21]. The Z-disk is involved in muscle force transmission and cell-signaling[22]. The Z-disk is linked to the extracellular matrix via costameres. Costameres connect the sarcomeres

to the sarcolemma through the dystrophin-glycoprotein complex and the integrin-vinculin-talin complex (whereby both complexes anchor on cytoskeletal actin-filaments) and subsequently to the extracellular matrix[22]. Costameres are located adjacent to the Z-disks and are not only responsible for bidirectional force transmission, but are also important for bidirectional cell-signaling and stabilization, and thereby the protection of the sarcolemma from muscle injury during muscle contraction[20,22].

Muscle fibers have multiple nuclei and are post-mitotic. Regeneration is possible via satellite cells located between the sarcolemma (cell membrane) and basement membrane which can differentiate into new muscle fibers or fuse with existing muscle fibers during hypertrophy or recovery[19,20,23]. The diameter of a single fiber measures about 100 µm[19]. Different muscle fiber types can be distinguished, the proportion of which varies in each muscle, depending on respective mechanical needs. The most common distinction is made between slow type I and fast type IIa and IIx fibers though, strictly speaking, more subtypes exist and some muscle fibers can simultaneously express more than one type of myosin heavy chain[19,21]. Many muscle fibers do not completely extend from tendon to tendon and instead often terminate on other muscle fibers[24]. Muscle fibers that do run to the tendon end at the aponeurosis, which continuously runs into the extramuscular tendon[24].

3.3. Intramuscular connective tissue

3.3.1. Importance of intramuscular connective tissue

Muscle fibers are surrounded by different layers of a three-dimensional intramuscular connective tissue (IMCT) network. The smallest connective tissue component is endomysium, which engulfs each individual muscle fiber[20,25,26] and thereby surrounds the individual cell membrane or sarcolemma[19]. The endomysium merges into the perimysium which surrounds muscle bundles. The perimysium merges into the epimysium which surrounds the whole muscle and merges into the fascia of the muscle that runs to the tendon. Between 1 to 10 % of muscle tissue is comprised of connective tissue, with the amount varying between muscle and fiber type[27]. The precise composition of intramuscular connective tissue is difficult to examine and is still under investigation[28]. Multiple collagens have to date been identified, primarily collagen I, III and V, although small amounts of collagen II, VI, IX, XI to XVI and XVIII to XIX have also been reported[27–29]. Collagen fibers are connected via cross-links which stabilize the collagen network[30]. Additional links are formed through advanced glycation end products (AGEs), resulting from a non-enzymatic reaction between collagen and glucose[30]. AGEs are postulated to be responsible for changing mechanical properties with age[30].

Connective tissue turnover is hereby generally controlled through matrix metalloproteinases and their specific inhibitors, e.g., an upregulation of IMCT production is needed after injury or during muscle development[31].

As a predominant component of muscle structure, intramuscular connective tissue has been postulated to be important for stability, transmission of shear forces, elasticity, as well as the nourishment of muscle fibers, and therefore essential to physiological muscle integrity and function[27,28,30,32]. IMCT has hereby been postulated to bear about five times the load compared to intracellular proteins[33]. IMCT also supports nerves and vessels of the muscle mechanically[27].

The stiffness of a muscle, which is also referred to as passive muscle tone or elasticity, is mostly influenced by connective tissue components of the extracellular matrix[28], which are stiffer than the muscle fibers themselves. Stiffness influences passive muscle tension which in turn controls muscle contractions and limits movements in the joints[34]. Very old muscle fibers are reported to show a remodeling of the ECM which would thus influence muscle function[27].

When examining single muscles fibers and muscle bundles compared to whole muscle, a non-linear increase in passive tension exists which is attributable to intramuscular connective tissue[35,36]. The passive modulus of connective tissue has been stated to be up to 25 times that of muscle fibers[37]. It has also been found that the lengthening of muscle fascicles only accounts for a maximum of half the lengthening of the muscle[24]. This can be partly attributed to changed pennation of muscle fibers, especially in very pennate muscles such as the soleus muscle, although the effect is small. Another explanation is the lengthening of the tendon and the aponeurosis, an effect which would possibly vary between muscles according to tendon length[24].

IMCT therefore has an indispensable role in skeletal muscle and deserves further attention.

3.3.2. Endomysium

Endomysium, as the smallest IMCT component, engulfs individual muscle fibers and is shared between neighboring muscle fibers[38].

Endomysium has a honeycomb[29] or mesh-up structure[28]. Its main collagenous components are collagens I and III[28,29]. Collagen fibers of the endomysium surround the muscle fibers, connect two neighboring muscle fibers, and run around and then connect capillaries and nerves to the muscle fibers[32]. Endomysium is connected to the sarcolemma via the basement membrane, which contains glycoproteins, such as laminin γ-1, and collagen type IV[28]. This connection is likely the location of force transmission, which primarily occurs through shear[28,31]. Force transmission hereby most likely occurs between adjacent muscle fibers within fascicles, where endomysium plays an important coordination role, most importantly between individual motor units[30,31]. Lateral load-sharing could explain how muscles can grow in that individual

muscle fibers can be excluded from the contraction process absent a general loss of contractile property[30].

Endomysium thickness changes during muscle contraction and adapts to changing muscle lengths. When a muscle contracts, endomysium becomes thicker; when a muscle stretches, endomysium becomes thinner[15]. Purslow et al. demonstrated that collagen fibers in the endomysial network reorient themselves with changing muscle length. While endomysium appears disordered and collagen fibers tend to have a circumferential orientation in resting muscle (short muscle length), the collagen fiber orientation becomes more longitudinal in lengthened muscle[30,38]. Increasing endomysium stiffness with increasing muscle length is predicted based on collagen reorientation[38].

3.3.3. Perimysium

The term perimysium is more loosely defined[28]. Perimysium surrounds muscle bundles and can generally be divided into primary perimysium surrounding primary fascicles and secondary perimysium surrounding larger secondary fascicles[30]. Perimysium is shared by two neighboring fascicles[30].

Contrary to endomysium, collagen fibers in perimysium appear more structured[28]. They run transversely to muscle fibers and show a crossed arrangement in two sets of perimysial sheaths[27,39]. Like endomysium, perimysium is mainly composed of collagen types I and III, although it has been postulated that the proportion between the two is not as evenly distributed as in endomysium, so that perimysium contains more type I collagen[28]. Elastin is mostly located in the perimysium of the muscle and potentially provides an elastic counterpart to the more rigid collagenous components[30].

Purslow et al. demonstrated that the amount of endomysium in a muscle is relatively stable, while the amount and distribution of perimysium varies significantly across different muscles[31]. This probably reflects the wide range of functions performed by different muscles and the resulting requirements on the connective tissue components[31].

3.4. Changes during immobilization

Muscle atrophy is a well-known result of unloading which occurs during immobilization, during spaceflight, and during bed rest[17,40]. Experimental bed rest with a 6° head-down tilt is recognized as an acceptable model for microgravity conditions and can be used as a study concept for muscle change during disuse[41]. Different anatomical muscles react differently to immobilization. Weight-bearing muscles are in particular strongly affected by gravitational loading and its loss during bed rest. Some aspects will be described in the following sections.

3.4.1. Effects on muscle function

It is well established that muscle function changes with training as well as with immobilization. The main function of skeletal muscle is to generate force and power through conversion of chemical to mechanical energy[19]. Muscle is further essential for basal energy metabolism as well as for the maintenance of blood sugar levels[19]. It is also the protein reservoir of the human body[42].

Muscle fibers atrophy, which results in functional impairments, such as the loss of muscle mass, the loss of peak force and power, an increased fatigue rate, and abnormal reflex patterns[3,17,43]. Long-term immobilization such as bed rest leads to a loss of strength which exceeds the loss of muscle mass[19,44], thereby suggesting the involvement of an additional contributing factor.

While the muscle's metabolic capacity increases with endurance training, this type of exercise does not affect force-generation or muscle size. Capillary density is much rather enhanced and the quantity of proteins involved in oxygen transport and aerobic glycolysis is increased. With resistance training, muscles hypertrophy and increase their force-generating capacity through increased protein synthesis[19]. These effects are roughly opposite during immobilization. After 6 months of spaceflight, for example, isometric maximal voluntary contractions decreased as did peak force[45]. After a 90-day bed rest study, jump height and peak force were reduced by 29.5 % and 25.8 %, respectively (after recovery day 4), in the control group[46].

3.4.2. Muscle fiber atrophy

Muscle atrophy is defined as the wasting of muscle tissue. It includes decreased muscle fiber size, cross-sectional area, and protein content, as well as the abovementioned functional impairments such as loss of force and power, increased fatigue rate, and increased insulin resistance[47–49]. Muscle atrophy hereby affects individual muscles differently, although both type I and type II fibers are affected[19]. During immobilization, muscle fibers type I generally atrophy more than muscle fibers type II[50], and anti-gravity muscles are more strongly affected[43]. In the triceps surae muscle, type I fibers of the soleus muscle atrophied the most, while type II fibers of the gastrocnemius muscle atrophied the least[45]. Extensor muscles atrophy at a faster rate than flexor muscles[43,45]. Atrophied muscle generally shows a fiber transition from slow to fast fibers[45] and atrophied muscle fibers become more reliant on glycogen and have a reduced capacity to metabolize fatty acids[43].

Different proteolytic systems are involved in muscle atrophy, including the autophagy-lysosome system and the ubiquitin-proteasome system[20,42,48]. Both systems are actively transcriptionally regulated by atrogenes, e.g., FoxO[48]. Lysosomes are acidic membrane-enclosed cell organelles which contain hydrolytic enzymes (proteases, nucleases, lipases). This system is important in maintaining homeostasis and degrades as well as recycles cell

components[48]. The ubiquitin-proteasome system works by enzymatic ubiquitination of proteins whereby proteins are marked for degradation via the proteasome (proteolytic breakdown of proteins into amino acids or polypeptides). The primary task of the ubiquitin-proteasome system in the muscle cell is the degradation of sarcomeric proteins with changing muscle activity[48,49]. This system is therefore highly selective for the marked proteins[42].

Apoptosis may play a role in muscle atrophy in addition to the autophagy-lysosome and the ubiquitin-proteasome systems. Apoptosis can generally be induced by various death-signals, such as reactive oxygen or nitrogen species. Death-signals induce reaction cascades resulting in the activation of enzymes, e.g., caspases. The cell is then fragmented into apoptotic bodies and phagocytized[51,52]. As muscle fibers are multinucleated, the number of nuclei increases or decreases with hypertrophy or atrophy, respectively[23,53]. This process likely occurs through apoptosis-like mechanisms and is most pronounced in slow-type fibers[23,51], although extensive discourse exists as to whether apoptosis occurs in myonuclei or only in the surrounding cells (satellite cells, fibroblasts, etc.)[54,55].

The rate of muscle fiber atrophy depends on the applied disuse model[45]. Muscle mass decreases in bed rest, e.g., a 90-day bed rest study found an up to 25 % decrease in calf muscle cross-sectional area[56]. Effects were already seen in shorter bed rest studies. A 5-day bed rest study found a decrease of 2 to 3 % in muscle cross-sectional area in the calf and thigh[57]. Muscle cross-sectional area increased rapidly in the first 14 days during recovery from bed rest, completely recovered after 90 days, and even showed an increase compared to baseline after 180 days[58].

It has been established that the basal protein synthesis rate initially decreases in disuse muscle atrophy models, and multiple molecular pathways have been found to influence the protein synthesis rate[45]. The results have been less clear for degradation and require additional investigation[45]. Fibers selectively loose contractile elements, which presumably contributes to loss of force and power. Actin is hereby lost proportionally more than myosin[43].

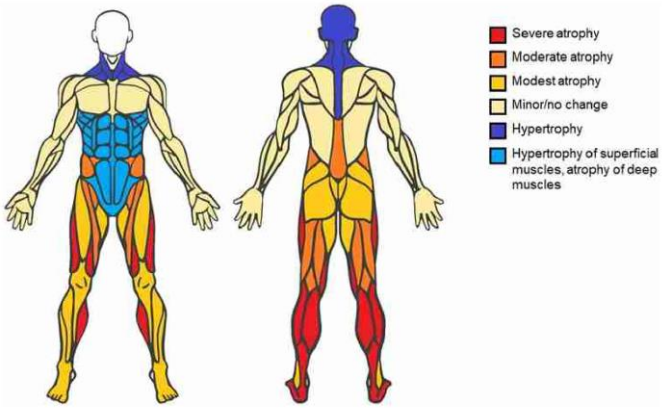

Figure 2 Atrophy and hypertrophy changes occurring in muscles during bed rest. The lower extremity, and especially the calf muscles, are strongly affected by atrophy.

3.4.3. Changes in intramuscular connective tissue

A major component present in the skeletal muscle is connective tissue. An additional factor must exist since the loss of strength exceeds the loss of muscle mass, as shown above. Because of the omnipresence of intramuscular connective tissue, it is reasonable to further study this component.

Williams and Goldspink conducted an experiment on the mouse soleus muscle and demonstrated an increased endomysium content[59]. They calculated the relation between intramuscular connective tissue area and muscle fiber area and could not exclude the possibility that the relative increase in endomysium was due to muscle fiber atrophy.

In rat immobilized muscle, it has been reported that the perimysium and endomysium of the intramuscular connective tissue appears to increase, and that in particular endomysium loses its normal structure. The increase in endomysial connective tissue is mostly attributed to an increase in collagen type III as well as a slight increase in collagen type I. The majority of the additional collagen fibers were found to directly surround the individual muscle fibers, increasing the separation between them. The density of the endomysial connective tissue increased in total. The density of the perimysial connective tissue also increased. The randomly oriented fibers hereby dominated, thereby preventing a clear pattern from being identified. The described alterations would possibly limit the individual muscle fibers' movement. [32]

Biochemically, IMCT increase or decrease is regulated by enzymes. Collagen biosynthesis enzyme activities decreased when rat hindlimb muscles were immobilized in casts[60]. Collagen degradation enzyme activities were shifted in the same direction[61]. Collagen expression is hereby also downregulated on the mRNA level[27].

A further hypothesis states that not only the amount of connective tissue, but also the type of collagen expressed in the IMCT, might change during disuse and affect muscle stiffness, however, no such transformation has to date been found[33].

It has further been shown that old rats have an impaired transmission of shear forces, comparable to dystrophic muscles[62]. In that an increase in collagen but no increase in collagen expression was observed, it was concluded that aging and the accumulation of extracellular matrix must be a consequence of a decrease in degradation of these proteins[62].

3.5. Relevance of muscle changes relating to IMCT in a clinical setting

Many diseases are in some degree associated with fibrosis, e.g., muscular dystrophies, diabetes, immobilization, and aging[28]. Fibrosis is hereby defined as an abnormal accumulation of connective tissue[37]. Spastic muscles are reported to have an increased collagen content[28] as well as abnormally increased muscle stiffness[34]. Conditions such as injuries, tumors, and

neurological disorders including multiple sclerosis, amyotrophic lateral sclerosis, stroke, or cerebral palsy, can lead to spasticity and contractures[33,34]. Spastic muscles can contracture and lead to abnormal joint positioning, resulting in major disability. A study on contractured hamstring muscles of patients with cerebral palsy found enhanced muscle stiffness as well as increased collagen content in affected muscles, thereby indicating a role of the extracellular matrix for passive muscle properties[63].

The effects occurring over a short time period during spaceflight are similar to the effects which are observed in aging over a longer time span. Since bed rest models mimic spaceflight conditions, the bed rest model can be used as an analog for aging[17]. Bed rest is therefore an accelerated model[50].

Aging and reduced physical activity are closely interrelated. It is to date not yet clear if the processes described in aging muscles are directly related to the aging process itself or are due to disuse or immobilization effects[19].

Sarcopenia, which the *European Working Group on Sarcopenia in Older People* defines as: 1) low muscle mass (less than 2 SD below the mean of a young reference group); and, 2) muscle weakness or reduced strength, affects between 4 and 27 % of the older population depending on study, country, and gender[19,45]. Aging muscle is shown to decrease in fiber number as well as in fiber size[64]. As in bed rest, muscle fibers type I atrophy significantly compared to type II fibers which undergo much less change[50]. A reduced myosin protein content and an increased stiffness were also observed[19]. Long-term bed rest generally induces similar changes to sarcopenia, most significantly the loss of muscle strength which exceeds the loss of muscle mass[19].

As set forth above, aging muscles have increased muscle stiffness. A study by Zimmermann et al. describes an increased amount of collagen cross-links during aging in animal studies which was reversable via exercise training[65]. No increase in enzymatically mediated cross-links was found in humans. Although an increase in AGEs existed, this most likely resulted from the life-long exposure to varying blood glucose levels[66]. Collagen cross-links presumably contribute to increased muscle stiffness which in turn affects the muscle's ability to generate force[66]. While it has been found that increased cross-linking can affect muscle stiffness, increased stiffness does not conditionally require increased cross-linking[33].

Pavan et al. examined young and old human vastus lateralis muscle and demonstrated increased passive tension associated to extracellular matrix as well as an increased connective tissue area with age. They concluded that increased stiffness in old age is caused by collagen accumulation[35]. In rats, endomysium, the smallest connective tissue component, increases early in life and again in senility, while otherwise remaining generally constant[67].

A mouse study on muscle stem cells found increased stiffness and altered ECM structure in old age. Muscle stem cells placed in an old ECM environment moreover led to increased gene-expression related to fibrogenesis, suggesting an accumulation of connective tissue though increased production[68]. A study of muscle from patients with cerebral palsy found a decreased amount of satellite cells. It was found that the absence of the satellite cells lead to an increased rate of fibroblast proliferation and thus to stiffer tissue production[33].

In addition to being relevant for space missions and for aging, bed rest models can provide insights and strategies of action for clinical usage in bed-bound and hospitalized patients and corroborate rehabilitation efforts.

3.6. Hypotheses and aim of this study

In summary, bed rest models can be used to investigate spaceflight-associated changes as well as aging processes where muscles atrophy. Intramuscular connective tissue is an integral component of skeletal muscle which influences active and passive functions and has to date been scarcely examined. IMCT changes during immobilization have to date also not been consistently examined. Many studies were conducted using animal models and are therefore only partly applicable to human skeletal muscle.

The main objective of the present study was to examine intramuscular connective tissue during long-term immobilization, and more specifically, the endomysium of the human soleus muscle. Histologically, ECM lies between two basement membranes of muscle fibers and can be measured via the space between two fibers. This can be accomplished using stains of proteins present in the basement membranes, such as laminin[33]. The difficulty lies in interpreting increased ECM space since this can change through an increase in ECM, but also through a decrease in fiber size, i.e., muscle fiber atrophy, or a combination thereof[33]. We nevertheless chose a histological approach since the exact biochemical composition of endomysium is not conclusively known[28].

Our study is based on the original mouse study by Williams and Goldspink[59]. As they found increased endomysium, they could not exclude effects of muscle fiber atrophy. Using a histological approach, differentiation between an absolute or relative intramuscular connective tissue increase is essential as muscle fiber atrophy in combination with unchanged endomysium content results in a relative connective tissue increase (see Figure 3).

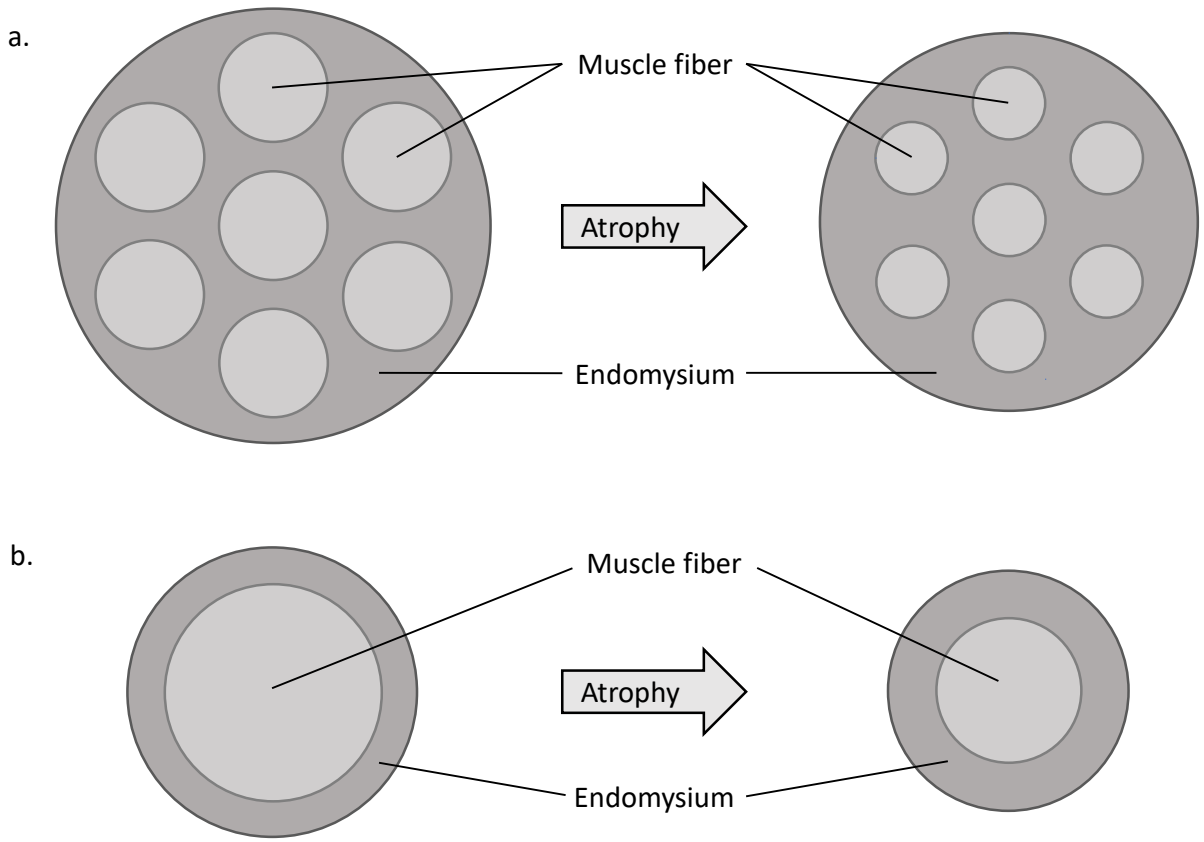

Figure 3 Visualization of the relative endomysium increase in the muscle bundle (a) and the muscle fiber (b) during muscle fiber atrophy. When muscle fibers shrink due to atrophy while the surrounding intramuscular connective tissue, i.e., endomysium, remains unchanged, the ratio between the two is shifted resulting in a relative connective tissue increase. This can be seen above via the increase of endomysium thickness around each individual muscle fiber.

We hypothesized: 1) that the endomysium would increase relatively during long-term bed rest in humans; and, 2) that this result would be attributable to muscle fiber atrophy and not to an absolute increase in endomysium content.

We also sought to explore the effect of artificial gravity as a countermeasure with regard to intramuscular connective tissue.

4. Publication

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SHORT COMMUNICATION

Effects of long-term immobilisation on endomysium of the soleus muscle in humans

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Abstract

Muscle fibres atrophy during conditions of disuse. Whilst animal data suggest an increase in endomysium content with disuse, that information is not available for humans. We hypothesised that endomysium content increases during immobilisation. To test this hypothesis, biopsy samples of the soleus muscle obtained from 21 volunteers who underwent 60 days of bed rest were analysed using immunofluorescence-labelled laminin γ-1 to delineate individual muscle fibres as well as the endomysium space. The endomysium-to-fibre-area ratio (EFAr, as a percentage) was assessed as a measure related to stiffness, and the endomysium-to-fibre-number ratio (EFNr) was calculated to determine whether any increase in EFAr was absolute, or could be attributed to muscle fibre shrinkage. As expected, we found muscle fibre atrophy ($P = 0.0031$) that amounted to shrinkage by 16.6% (SD 28.2%) on day 55 of bed rest. ENAr increased on day 55 of bed rest ($P < 0.001$). However, when analysing EFNr, no effect of bed rest was found ($P = 0.62$). These results demonstrate that an increase in EFAr is likely to be a direct effect of muscle fibre atrophy. Based on the assumption that the total number of muscle fibres remains unchanged during 55 days of bed rest, this implies that the absolute amount of connective tissue in the soleus muscle remained unchanged. The increased relative endomysium content, however, could be functionally related to an increase in muscle stiffness.

KEYWORDS
connective tissue, endomysium, immobilisation, muscle atrophy

1 | INTRODUCTION

Muscle atrophy is a well-known result of unloading which occurs during immobilisation, spaceflight and experimental bed rest (Booth, 1994; Pavy-Le Traon et al., 2007), where muscle tissue is not used in its normal capacity. The need to understand the underlying mechanisms of muscle atrophy increases with an ageing society, and also with ambitions to travel to Mars. Experimental bed rest with a 6° head-down tilt is widely recognised as an acceptable model for microgravity conditions and has been used to investigate muscle change during disuse (Hargens & Vico, 2016; Widrick et al., 1999). Bed rest-induced muscle wasting occurs as a result of reduced myofibrillar protein synthesis (Gibson et al., 1987), and the resulting atrophy is most pronounced in the calf musculature, especially in the soleus muscle (Belavý et al., 2009).

Many different countermeasures (predominantly physical exercise regimes) have been examined in an attempt to prevent physical deconditioning during spaceflight. Making use of artificial gravity as a more generalised countermeasure may be needed in order to safely perform deep-space missions. With regards to skeletal muscle, it has already been shown that performing resistance exercise training on a short-arm centrifuge is feasible (Yang et al., 2007) and that it can at least partly prevent muscle deconditioning during experimental bed rest (Caiozzo et al., 2009; Edmonds et al., 2008). Hence, the Artificial Gravity Bed Rest Study with the European Space Agency (AGBRESA) was devised to assess the effectiveness in mitigating bed rest-induced deteriorations in the fields of cardiovascular, immunological, genetic, muscle and bone, ocular, vestibular, psychological, sleep, cognition and behavioural performance, and the present sub-study makes use of biopsy samples that were harvested from the soleus muscle within the AGBRESA study.

While it is established that muscle fibre atrophy leads to functional impairments, such as a loss of peak force and power (Fitts et al., 2010; Fitts et al., 2001), the changes transpiring within the muscular connective tissue have largely been neglected. As recently reported, the mechanical properties at whole-muscle level are at least partly influenced by connective tissue, and intramuscular connective tissue should therefore receive more attention. The smallest connective tissue component is the endomysium, which engulfs each individual muscle fibre (Borg & Caulfield, 1980; Turrina et al., 2013) and surrounds the individual cell membrane, or sarcolemma (Frontera & Ochala, 2015). As reviewed by Gillies and Lieber, endomysium has a mesh-up structure composed of equal amounts of collagen types I and III. It is interlinked with the muscle basement membrane which is in turn interlinked with the sarcolemma. The basement membrane consists mainly of type IV collagen and glycoproteins, such as laminin γ-1 (Gillies & Lieber, 2011). It has been postulated that intramuscular connective tissue is important for stability, force transmission, elasticity, as well as the nourishment of muscle fibres, and is therefore essential to physiological muscle integrity and function (Gillies & Lieber, 2011; Järvinen et al., 2002; Kjaer, 2004).

Research on this omnipresent component in skeletal muscle during muscle disuse is, however, scarce. In rat, the endo- and perimysium was increased and had lost its normal ultra-structure upon immobilisation (Järvinen et al., 2002). It has further been demonstrated in mice that immobilisation results in an increased endomysium content of the soleus muscle (Williams & Goldspink, 1984). One has to consider, though, that the latter study merely considered the relation between connective tissue and muscle fibre size, and that it does not exclude the possibility that the relative enrichment in endomysium is foremost attributable to fibre atrophy. Savolainen et al. have observed decreases in collagen biosynthetic enzyme activities in cast-immobilised rat hindlimb muscle (Savolainen et al., 1987), but increases in those enzyme activities when the hindlimb muscles were denervated (Savolainen et al., 1988a). However, intramuscular collagen degradative enzyme activity is shifted in the same direction as collagen synthetic enzyme activity (Savolainen et al., 1988b), and *ex vivo* enzyme activity does not necessarily reflect *in vivo* enzyme turnover. Moreover, the turnover of collagen is only 1–2%/day in rat skeletal muscle, whereas the fractional synthesis rate of muscle non-collagenous proteins is 12% per day (Reeds et al., 1980), all of which may explain why the total content of hydroxyproline per muscle, as a surrogate for collagen mass within an anatomical muscle, was fairly constant in another rat immobilisation study (Karpakka et al., 1991), and also in the aforementioned studies (Savolainen et al., 1987, 1988a, 1988b).

Based on the findings of Williams & Goldspink (1984), we hypothesised that the relative amount of intramuscular connective tissue in the human soleus muscle increases during long-term bed rest, which is solely attributable to muscle fibre shrinkage rather than an increase in the absolute amount of intramuscular connective tissue. The increase in the relative amount of intramuscular connective tissue could contribute to reported disproportional loss of muscle mass and muscle strength and thereby provide a step towards enhancing our understanding of the impaired function of an atrophied muscle.

New Findings

- **What is the central question of this study?**
  While muscle fibre atrophy in response to immobilisation has been extensively examined, intramuscular connective tissue, particularly endomysium, has been largely neglected: does endomysium content of the soleus muscle increase during bed rest?
- **What is the main finding and its importance?**
  Absolute endomysium content did not change, and previous studies reporting an increase are explicable by muscle fibre atrophy. It must be expected that even a relative connective tissue accumulation will lead to an increase in muscle stiffness.

2 | METHODS

2.1 | Ethical approval

The study was approved by the Ethics Committee of the North Rhine Medical Association (Ärztekammer Nordrhein, reference number 2018143) in Düsseldorf, Germany, and was registered in the German Clinical Trials Register (DRKS-ID: DRKS00015677). All subjects provided written informed consent prior to their participation in the study. The study conformed to the standards set by the *Declaration of Helsinki*.

2.2 | Study design

A group of 24 healthy subjects (16 men, 8 women, 33 ± 9 years; 175 ± 9 cm; 74 ± 10 kg) participated in the 60-day AGBRESA study, which was conducted jointly by the German Aerospace Center, the European Space Agency and the National Aeronautics and Space Administration. AGBRESA's objective was to determine the effects and effectiveness of short-duration continuous (cAG) and intermittent centrifugation (iAG), i.e., artificial gravity, as a countermeasure to the negative effects of immobilisation and disuse. Subjects were pseudo-randomly assigned to the passive control (Ctrl) or to an intervention group. Subjects underwent extensive medical and psychological evaluation prior to being included in the study. More detailed information is set forth in *Guidelines for standardisation of bed rest studies in the spaceflight context* (Sundblad & Orlov, 2014). Details of the AGBRESA study have been published which specify the daily routines (Frett et al., 2020).

2.3 | Biopsy acquisition

Biopsies were obtained from the soleus muscle of 21 of the AGBRESA subjects (14 men, 7 women) under sterile conditions at baseline (BDC), at day 6 and at day 55 head down tilt bed rest (HDT6 and HDT55). Acquisition followed skin disinfection and local anaesthesia with lidocaine. Muscle tissue was extracted using a rongeur (diameter of 4 mm) which extracted approximately 150 mg of muscle tissue. Muscle biopsy specimens were mounted with Tissue-Tek O.C.T. Compound (Sakura, Torrance, CA, USA) with a fibre orientation adjusted for transversal sectioning and immediately shock-frozen in liquid nitrogen and stored at −80°C. Frozen, unfixed muscle biopsies were sectioned at −20°C on a cryostat (Leica CM 1850 UV; Leica Biosystems, Wetzlar, Germany). Slices of 8 μm were transferred to adhesive microscope slides for myopathological analysis and stored at −20°C until staining.

2.4 | Staining protocol

Indirect immunofluorescence staining was conducted at room temperature in a humid environment protected from light. Slides were let to equilibrate to room temperature, fixed in formaldehyde prepared from 0.5% paraformaldehyde, and washed three times in phosphate-buffered saline (PBS) for 5 min and briefly in distilled water. Slides were subsequently blocked with 5% normal goat serum in PBS/0.2% Tween-20 for 30 min. Anti-laminin γ-1 antibody (polyclonal rabbit anti-mouse laminin γ-1 chain, Immundiagnostik, Bensheim, Germany, cat. no. AP1001.1, RRID:AB_227233) at a dilution of 1:200 in blocking buffer was applied to the slides and allowed to incubate for 1 h. After washing in PBS three times, the slides were incubated with the secondary antibody (polyclonal Cy2-conjugated AffiniPure goat anti-rabbit antibody, Jackson ImmunoResearch Laboratories, West Grove, PA, USA, cat. no. 111-225-144, RRID:AB_2338021) at a dilution of 1:500 in blocking buffer for 45 min. After incubation, the slides were washed in PBS three times, washed briefly in distilled water, mounted with ProLong Gold Antifade Mountant with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific, Waltham, MA, USA, P36941), and stored at 4°C.

2.5 | Image analysis

Tiled images were captured using a Zeiss Axio Observer.Z1 microscope (Carl Zeiss Microscopy, Oberkochen, Germany) with a ×25 objective (NA 0.8). The observer was blinded to the operational day and group allocation when analysing the images. Laminin γ-1-stained images were stitched and analysed using Zeiss ZEN Desk 3.1 software. Each measurement frame (called region of interest (ROI) for readability) included at least 50 muscle fibres, and ROIs were carefully placed within the stitched image, i.e., the stained section, to exclude artefacts and perimysium, thereby containing muscle fibres and endomysium and its enclosures (capillaries, nerve fibres) only. For each muscle fibre that is bordered by its laminin γ-1-positive basement membrane, the minimal and maximal Feret-diameters, muscle fibre perimeter ($Peri_{Fibre}$) and muscle cell area (A_{Fibre}) were computed with Zeiss ZEN software (version 3.1, blue edition). The endomysium space is hereby delineated by the laminin γ-1-stained basement membranes of neighbouring muscle fibres. We assume that endomysium area is correlated with endomysium content and therefore used endomysium area as a parameter to estimate endomysium content. Notably, the ROIs' borders contained truncated fibres. Hence, in order to adjust for truncation effects upon endomysium readouts, fibres adjacent to the border were identified being away from the border by less than the median of half the maximal Feret diameter. The validity of this approach was confirmed by visual inspection with custom-made R scripts (R Foundation for Statistical Computing, Vienna, Austria). For statistical analyses, diameter, cross-section and perimeter values were averaged over only fibres that were not truncated by the ROI borders.

Next, we assessed, the endomysium-to-fibre-area ratio (EFAr). To this purpose, total fibre area (A_{Tot_Fibre}, which includes border-adjacent and thus truncated fibres) was computed, and the total endomysium area (A_{Tot_Endo}, visible as the area delineated by two laminin γ-1-positive basement membranes, see Figure 1) was computed as the difference between the ROI's area and A_{Tot_Fibre}, and EFAr was

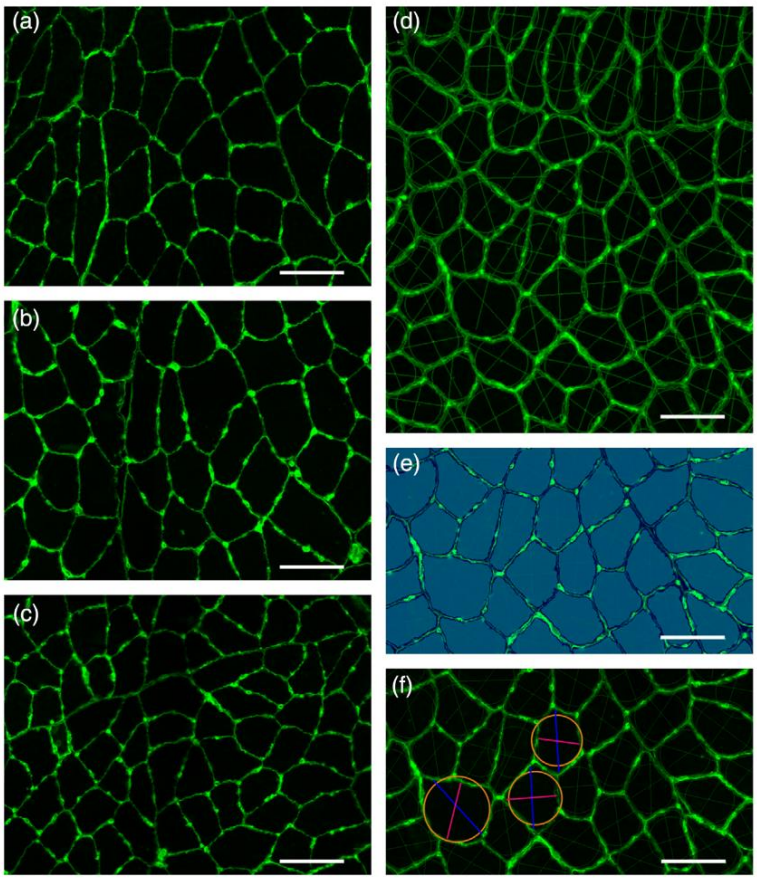

FIGURE 1 (a–c) Typical staining from one representative subject before (BDC, a), at day 6 (HDT6, b) and at day 55 (HDT55, c). (d) Segmentation of muscle fibres shown in a part of the region of interest (ROI) in a biopsy. (e) The area of endomysium if the muscle fibre area (coloured) is subtracted. The model used assumed that muscle fibres are approximately round and can be approximated by using parameters of the minimal (red) and maximal (blue) Feret-diameters (f). Scale bars 100 µm

calculated as a percentage as:

$$EFAr = 100 \cdot \frac{A_{Tot\_Endo}}{A_{Tot\_Fibre}}$$

We specifically chose to assess the endomysium-to-fibre-area ratio, and not the fractional area of the endomysium within the entire ROI, firstly because this describes the ratio of passive-to-active constituents of muscle tissue, and secondly because this measure yields a stronger contrast to the measure of endomysium-to-fibre number (see below).

Endomysium thickness was calculated, using the summed perimeters of all fibres (ΣPeri, including border-adjacent fibres) as:

$$\frac{A_{Tot\_Endo}}{0.5 \cdot \Sigma Peri - Peri_{ROI}}$$

where $Peri_{ROI}$ is the ROI's perimeter. The factor 0.5 in the denominator is necessary to account for the fact that each endomysium strut is outlined by two muscle fibre perimeters, and subtraction of $Peri_{ROI}$ adjusts for the border-truncated fibres.

For computation of the endomysium-to-fibre-number ratio (EFNr), we also had to take care of the border-truncated fibres. It turns out that this is well feasible by adjusting the fibre number for the border-adjacent fibres of the ROI ($N_{Border\_Adjusted}$)

$$N_{Border\_Adjusted} = \frac{A_{Tot\_Fibre\_Border}}{A_{Fibre\_Mean}}$$

where $A_{Tot\_Fibre\_Border}$ is the total area of all border-adjacent fibres and A_{Fibre_Mean} is the average area of all fibres not adjacent to the ROI border. Accordingly, EFNr was computed as

$$EFNr = \frac{A_{Tot\_Endo}}{N_{Non\_Border} + N_{Border\_Adjusted}}$$

where N_{Non_Border} is the number of fibres that are entirely located within the ROI.

2.6 | Statistical analyses

Statistical analyses were performed using R version 3.6.1. All calculations were performed using an analysis of variance with a linear mixed-effects model. Results are shown as means and standard deviations. The level of significance was set at $P < 0.05$. Four

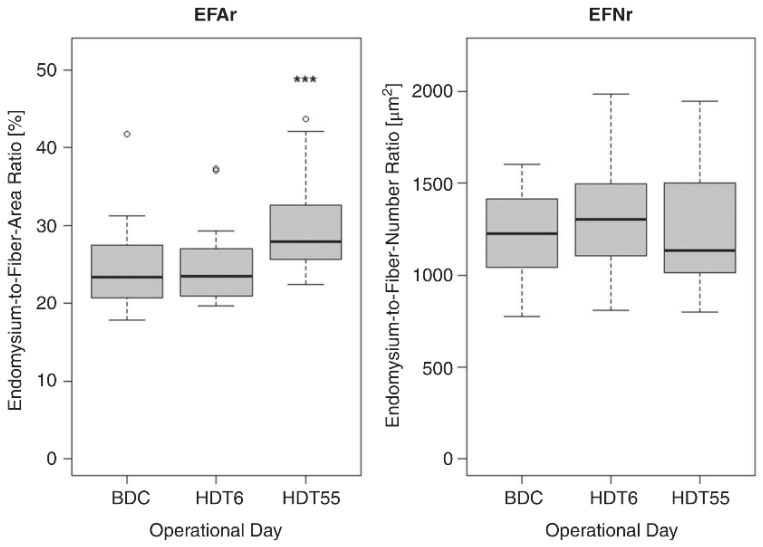

FIGURE 2 Summary of results from all biopsies. Endomysium per muscle fibre area before (BDC) and after 6 (HDT6) and 55 (HDT55) days of head-down tilt bed rest (a), as well as endomysium per muscle fibre number (b)

TABLE 1 Anthropometrics for the control (Ctrl) and intervention groups (iAG and cAG) at baseline

Variable	Ctrl	iAG	cAG	All
Number of subjects	6	7	8	21
Age (years)	35.5 (7.9)	34.9 (11.1)	31.9 (9.7)	33.9 (9.4)
Height (cm)	175.7 (8.0)	177.3 (10.8)	172.5 (8.0)	174.3 (8.7)
Weight (kg)	75.0 (11.3)	71.4 (4.9)	71.8 (10.2)	72.6 (8.8)

Values are means ± SD.

biopsies were excluded from analysis due to poor biopsy quality or an insufficient number of analysed fibres (<50 fibres).

3 | RESULTS

There were no significant differences at baseline across groups in either anthropometric characteristics or in biopsy data (all $P > 0.20$, see Table 1).

3.1 | Muscle fibre atrophy

We first analysed the fibre area (see Table 2) to determine the presence of muscle fibre atrophy. We found a significant effect of time (BDC, HDT6 and HDT55) pertaining to the decrease of muscle fibre area ($F(2,34) = 6.9$ and $P = 0.0031$), but no interaction between group (iAG, cAG or Ctrl) and time ($F(2,34) = 1.03$ and $P = 0.41$). Following up the time effect by *a priori* contrasts revealed a shrinkage of 16.6% (SD 28.2%) at HDT55 ($P = 0.028$), but no change at HDT6 ($P = 0.90$). The minimal Feret-diameter showed a main effect for time ($F(2,34) = 8.42$ and $P = 0.0011$), but not for the interaction between time and group ($F(4,34) = 0.67$ and $P = 0.62$). The main effect for time was again significant at HDT55 ($P < 0.001$), but not at HDT6 ($P = 0.93$).

3.2 | Intramuscular connective tissue

Endomysium-to-fibre-area ratio (EFAr, Table 2) showed a main effect of time ($F(2,34) = 10.4$ and $P < 0.001$), but no group-by-time-interaction ($F(4,34) = 1.07$ and $P = 0.39$). *A priori* contrast testing showed that EFAr was increased on day HDT55 ($P < 0.001$, see Figure 2a), but not on day HDT6 ($P = 0.43$). We furthermore computed the thickness of the intramuscular connective tissue. There was no main effect of time ($F(2,34) = 2.30$ and $P = 0.12$), and also no interaction between time and group ($F(4,34) = 1.56$ and $P = 0.21$). Likewise discordant from the EFAr, there was no effect of time for EFNr ($F(2,34) = 0.48$ and $P = 0.62$, Figure 2b), and also no interaction effect between time and group ($F(4,34) = 0.79$ and $P = 0.54$).

To conclude, we did not find any group effects in any outcome variable (muscle fibre atrophy, EFAr, endomysium thickness and EFNr).

4 | DISCUSSION

The purpose of this study was to determine whether endomysium content, assessed through the endomysium area, increases during long-term immobilisation, and whether this increase could possibly be a missing factor in explaining the seemingly exaggerated loss in muscle function compared to muscle atrophy in disuse models. In line with the hypotheses, results showed an increase in the relative amount of intramuscular connective tissue, but no increase in the absolute amount of endomysium during immobilisation. As the absolute amount of connective tissue remained unchanged, but fibres atrophied, the relative thickness of endomysium increased.

TABLE 2 Muscle fibre morphology and intramuscular connective tissue before (BDC) and after 6 (HDT6) and 55 (HDT55) days of head-down tilt bed rest for the control (Ctrl) and intervention groups (iAG and cAG)

Variable	Group	BDC	HDT6	HDT55
Fibre CSA (μmš)	Ctrl	6011 (2962)	6255 (1951)	4104 (1526)
	iAG	4983 (991)	5037 (989)	4074 (2006)
	cAG	5150 (962)	4978 (1272)	4758 (1895)**
	All	**5340 (1734)**	**5362 (1465)**	**4336 (1753)**
Feret Min (μm)	Ctrl	69.5 (14.2)	69.7 (7.9)	57 (8.4)
	iAG	63.1 (5.8)	63.1 (5.8)	55.6 (15.5)
	cAG	63.6 (6.8)	64 (9.1)	59.5 (13.1)
	All	**65.1 (9.2)**	**65.3 (7.9)**	**57.5 (12.1)*****
EFA ratio (% of fibre CSA)	Ctrl	21.9 (3.6)	23.4 (3.6)	30.7 (6.1)
	iAG	24.2 (2.6)	26.1 (6.0)	34.4 (12.7)
	cAG	27.1 (7.4)	25.8 (5.3)	28.7 (7.3)
	All	**24.6 (5.4)**	**25.2 (5.0)**	**31.1 (8.9)*****
Endomysium thickness (μm)	Ctrl	8.73 (3.77)	9.45 (2.06)	9.18 (1.22)
	iAG	8.55 (1.42)	9.32 (2.71)	10.17 (1.56)
	cAG	9.78 (2.08)	8.99 (1.3)	9.23 (1.49)
	All	**9.07 (2.45)**	**9.23 (1.97)**	**9.51 (1.43)**
EFN ratio (μmš/fibre)	Ctrl	1369 (928)	1439 (370)	1203 (282)
	iAG	1201 (249)	1306 (372)	1212 (354)
	cAG	1342 (195)	1252 (250)	1295 (382)
	All	**1303 (503)**	**1323 (322)**	**1240 (328)**

Values are means ± SD. Fibre area given as cross-sectional area (CSA), and minimal fibre diameter as Feret Min. Significances from baseline:
**$P < 0.01$
***$P < 0.001$.

Naturally, the present technical approach has not directly assessed the constituents of endomysium, but rather the limits of the endomysium space (defined by the laminin γ-1-stained basement membranes). However, to do so would be difficult as the exact biochemical composition of endomysium is generally not well established (Gillies & Lieber, 2011), and it is possible that single constituents, e.g., certain collagens, are differentially expressed during the atrophy response. In keeping with the primary aim of our study, we therefore approached the endomysium space histologically.

The endomysium-to-fibre-number ratio in this study was calculated to unambiguously determine whether previously reported increases in endomysium content with immobilisation are solely an effect of muscle fibre atrophy, or whether it is attributable to an actual net gain in intramuscular connective tissue. Since an increase was only shown in endomysium-to-fibre-area and not in endomysium-to-fibre-number, the results clearly demonstrate that the previously reported increase in endomysium content with disuse is most likely an effect of muscle fibre shrinkage. Williams and Goldspink did in fact calculate the endomysium-to-fibre-area ratio between day 1 and 3 weeks of immobilisation (Williams & Goldspink, 1984) and concluded that a direct effect of muscle fibre atrophy could not be excluded. If indeed the endomysium content per fibre remains constant, and if the number of muscle fibres in a given muscle remains likewise unaffected by bed rest, then one would expect the total collagen content to be unchanged in atrophied rat muscles mentioned above (Savolainen et al., 1987, 1988a, 1988b). It is of interest in this context that muscle collagen synthesis rate is comparable to muscle myofibrillar protein synthesis rate, but lower than tendon collagen synthesis rate in ambulatory younger and older people (Babraj et al., 2005). Under steady state conditions, one would expect, accordingly, that protein breakdown rates are comparable between myofibrillar proteins and muscle collagen. Immobilisation-related fibre atrophy is mostly due to reductions in myofibrillar protein synthesis rate, with protein breakdown being mainly unaffected (Paddon-Jones et al., 2006), and tendon collagen synthesis reductions in limb immobilisation seem to match the reductions in myofibrillar protein synthesis (de Boer et al., 2007). Therefore, if indeed the endomysium content of the muscle remains constant in bed rest, there would have to be a reduction in endomysium protein breakdown that is commensurate to reductions in endomysium protein synthesis.

An increase in the endomysium content has also been hypothesised to take place during other circumstances, for example, ageing and spasticity (Kjaer, 2004). Many different muscle disorders include muscle fibrosis, meaning an abnormal accumulation of connective tissue (Lieber & Ward, 2013). Although the consequences

of exaggerated endomysium content remain to be elucidated in general, it has been found that the enhanced stiffness in contractured hamstring muscles of patients with cerebral palsy is attributable to enhancement of extracellular matrix content (Smith et al., 2011), and very similar effects have been demonstrated for older age (Pavan et al., 2020). Ward et al. (2020) demonstrated that passive modulus increases non-linearly when examining properties of single fibres, fibre bundles, fascicles and whole muscles, indicating the role of the extracellular matrix. Of note, generation of force and of stiffness are two different mechanical functions of muscle (Lai et al., 2019). Whilst strength, which is related to the sarcomeres-in-parallel, is necessary to generate force, and thereby provides movement ability, stiffness, which is related to connective tissue, allows elastic storage and return of energy. Extracellular matrix of the muscle is assumed to have a modulus up to 25 times that of muscle fibres (Lieber & Ward, 2013). Future studies should therefore address the question of whether the relative enrichment of endomysium and the other constituents of muscular connective tissue do indeed affect muscle strength and stiffness differentially.

In conclusion, we found that the endomysium of the intramuscular connective tissue does not increase in relation to fibre number, but is enhanced in relation to fibre size in response to long-term immobilisation in humans. These changes must be expected to differentially affect the muscle's abilities to generate force or to store elastic energy, thereby potentially increasing the relative stiffness of the atrophied skeletal muscle.

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COMPETING INTERESTS

Authors declare no competing interests.

AUTHOR CONTRIBUTIONS

J.R. conceived and designed the study. G.K.T., C.B., I.G., J.R. and B.G. performed and processed the muscle biopsies. G.K.T. immunostained the samples. Y.L. provided support on microscopic settings. G.K.T. and J.R. analysed the data. C.S.C. participated in final data presentation. All authors contributed to the interpretation of data. G.K.T. drafted the manuscript. C.S.C. supported figure preparation. All authors contributed to the revision of the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT

Data will be made available upon reasonable request to the corresponding author.

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5. Discussion

5.1. Summary of results

The object of our study was to determine the effects of long-term immobilization on the intramuscular connective tissue of the human soleus muscle. We hypothesized that endomysium would increase relatively during bed rest.

To briefly summarize the results, we observed muscle fiber atrophy expressed as a decrease in muscle fiber cross-sectional area as well as a decrease in the minimal Feret diameter on day 55 of bed rest compared to before bed rest. We further observed increased endomysium content per fiber area on day 55 of bed rest. However, when we determined the endomysium content in relation to muscle fiber number, this ratio remained unaffected by bed rest. Only an increase in both parameters would have indicated an absolute increase of intramuscular connective tissue. As only endomysium-to-fiber-area increased while endomysium-to-fiber-number remained unchanged, the observed increase in endomysium was concluded to be an effect of muscle fiber atrophy – to the extent bed rest triggered no generation of new muscle fibers (which appears a fair assumption to make). We concluded that the thickness of endomysium thus increased as muscle fibers themselves atrophied.

These findings agree with our primary hypothesis.

5.2. Internal validity

We examined muscle fiber atrophy via fiber cross-sectional area as well as minimal Feret diameter and calculated the endomysium-to-fiber-area ratio, endomysium-to-fiber-number-ratio, and endomysium thickness.

We hereby approached endomysium histologically and did not determine any qualitative changes in the endomysial connective tissue network. As noted by Lieber and Fridén, the histological endomysial space is difficult to interpret as it can be influenced by increasing ECM, decreasing fiber size, or a combination thereof[33]. We found muscle fiber atrophy as expected during long-term immobilization. To exclude effects of muscle fiber atrophy on endomysium-to-fiber-area, we further determined the endomysium area in relation to muscle fiber number. To avoid any distortion by non-endomysial intramuscular connective tissue, i.e., perimysium, analyzed sections were chosen to only include endomysium. We furthermore only included biopsies with a minimum of 50 fibers in the analyzed section. Laminin γ-1 was solely used as a marker for basement membrane as the border between muscle fibers and endomysial space. Luminescence was hereby not used for quantification. We instead opted for a histological approach in that it has not been conclusively determined which IMCT components, e.g., collagens, are expressed in endomysium and perimysium and if their expressions differ during the immobilization response[28].

We sought to increase internal validity by having subjects randomly assigned to the control or intervention group, as well as by blinding the observer to the operational day and group allocation when performing the study. Subjects strictly adhered to the study protocol, including control of the 6° HDT position at all times, calculated nutritional intake, and a daily routine. As our study was based on the extraction of biopsies and the analysis thereof, results did not rely on the subjects' cooperation during repetitive measurements.
The observed results are deemed plausible in that they coincide with our hypothesis and the above-mentioned steps were undertaken to maximize validity.

5.3. Comparisons to previous findings

5.3.1. Animal immobilization studies

Animal studies have extensively been used as a model to examine immobilization effects on muscles. Hindlimb unloading in rodents was hereby primarily established as a microgravity analog to investigate musculoskeletal functions during spaceflight[69].
The original work by Williams and Goldspink on mice determined an increased endomysium content in the soleus muscle after immobilization. The analyzed time span ranged from one day to three weeks. They thereby calculated the ratio of endomysium to muscle fiber area and found an increase in the determined value[59]. Because we intended to examine intramuscular connective tissue in the human soleus muscle, we also calculated the endomysium-to-fiber-area ratio and determined an increase. We additionally calculated the endomysium-to-fiber-number ratio and could therefore determine that the IMCT increase during long-term immobilization is relative.
Järvinen et al. visualized the connective tissue network in rats using scanning electron microscopy. They observed an increase in endomysium and perimysium as well as a disturbance of the normal endomysial structure[32], no quantification of the visualized connective tissue was, however, performed.
Jozsa et al. conducted a rat hindlimb-immobilization study with the analyzed time ranging from one to three weeks which also described increased endomysial and perimysial connective tissue after immobilization. The soleus muscle thereby showed a marked relative increase in the IMCT proportion after one week of immobilization, which matches with our findings. Connective tissue hereby increased more prominently when the muscle was immobilized in a lengthened position compared to a shortened one. Jozsa et al. proposed that the differing IMCT proportions in different positions could be explained through increased vulnerability of muscles containing primarily type-I muscle fibers. A decrease in capillary density also occurred[70]. While this aspect was not examined by us, a decrease in capillary density seems a logical consequence in that atrophied muscle fibers require less nourishment.

Hindlimb immobilization rat studies found that, during immobilization atrophy, decreased biosynthetic enzyme activity leads to reduced collagen synthesis, while during denervation atrophy, the rate of collagen synthesis increases via increased enzyme activities[60,71]. Degradation enzyme activity also increased, which suggests muscle atrophy as a regulator for intramuscular collagen metabolism[61]. Analysis of enzyme activities showed very slow turnover rates for collagen, which suggested that an increase in collagen content during immobilization is only caused by higher degradation of non-collagenous components and that no absolute fibrosis occurs[72]. The rate of collagen turnover is generally much slower than that of non-collagenous components (1-2 % per day compared to 12 % per day)[73], which could explain the finding of fairly stable collagen contents in all of the above-mentioned studies. Collagen content was hereby measured biochemically via hydroxyproline. As our study did not examine individual IMCT components or enzyme activities, no statement on qualitative changes is possible. Our finding of a relative increase in endomysium would concur with a stable collagen content during immobilization. Collagen enzyme activities would need to shift analogously to maintain a stable state.

In summary, results from animal studies generally concur with our findings of a relative increase in endomysium content.

5.3.2. Human bed rest studies

Multiple bed rest studies have been conducted with the objective of examining muscle response and of developing an effective countermeasure against spaceflight and immobilization induced responses. Studies mostly focused on the muscle fibers (via a variety of methods) and functional parameters. The results thereof will be discussed below.

Muscles of the lower extremity have been found to atrophy the most. We found that the soleus muscle had atrophied by 16.6 % by day 55. This is in line with other bed rest studies that show similar muscle atrophy rates[44,56,58].

A 60-day bed rest study testing a jump training intervention protocol of about three minutes showed no significant decreases in muscle mass, maximal leg strength, or peak oxygen uptake in the intervention group, while the control group showed significant decreases in all of the above-mentioned parameters. The study also reported the previously described higher loss of muscle strength compared to muscle mass. The loss of muscle mass measured via dual energy X-ray absorptiometry (DXA) amounted to 5 %, while the loss in knee extensor maximal voluntary contraction (MVC) was about 40 %. High intensity jump training proved to be an effective integrated countermeasure in preserving musculoskeletal and cardiovascular functions[74]. Another five-week bed rest study examined muscle area and muscle strength. Muscle area was measured using magnetic resonance imaging (MRI). A proportionally higher loss in muscle mass compared to muscle strength was thereby measured. The triceps surae

mass hereby decreased about 12 %, while maximal strength decreased by 26 %. It was also demonstrated that muscles atrophy differently, i.e., losses in the plantar flexors are increased compared to losses in the dorsiflexors[44]. These long-term bed rest studies show similar scales of muscle atrophy as were determined via our muscle biopsies. While we did not measure and therefore cannot correlate any strength parameters with biopsy results, it can be assumed that these would also show similar decreases in muscle strength.

During recovery after a 90-day HDT bed rest study, calf muscle cross-sectional area showed gains in comparison to before bed rest. This is most likely not only due to muscle fiber hypertrophy, but also due to an increase in non-contractile elements[58]. This response to reloading should be examined further in relation to the IMCT response. It might thereby be necessary to take additional biopsies during recovery.

A short-duration bed rest study of 5 days found decreased muscle cross-sectional area in the plantar flexors using MRI. MVC decreased in the control group, but was maintained in the standing intervention group, and increased in the locomotion replacement training group. The study found no effect of 5-day bed rest on peak force. The loss of muscle cross-sectional area of 2-3 % after 5 days hereby corresponds with previous findings on muscle atrophy during immobilization[57]. Standing for 25 minutes did not prevent loss in CSA, and losses were the same for the control and for the standing group. This agrees with our finding that artificial gravity could not prevent muscle fiber atrophy. The BRAG-1 5-day HDT-bed rest study postulated that daily exposure to artificial gravity would not counteract muscular effects of bed rest[75], and this was shown to be true. An increase in g-forces on the short-arm centrifuge or an addition of training regimes could increase efficacy of artificial gravity[75].

Intramuscular connective tissue changes during bed rest were also analyzed. Collagen content of the soleus muscle was found to not significantly change after 90 days of bed rest, neither in the resistance training intervention group, nor in the control group. Results could, however, be skewed for the soleus muscle in that exercises were squat-based which target plantar flexors less. No differentiation between endomysium and perimysium exists in that a biochemical approach was used to determine collagen content[76]. Further analyses should therefore attempt to differentiate between the endomysial and perimysial components. This is especially difficult with biochemical methods as preparations must be performed delicately to preserve both IMCT components for analysis.

In summary, we found muscle fiber atrophy as previously described in human bed rest studies of the lower extremity. Limited research has been performed on IMCT. IMCT therefore deserves additional attention, which is one of the reasons we chose to focus on IMCT. Potential functional implications, in particular regarding muscle stiffness, will be discussed below.

5.3.3. Stiffness

While an association between muscle stiffness and the extracellular matrix has been found, it has not yet been determined if the underlying cause of muscle stiffness is an increase in individual ECM components or a structural change. Increased muscle stiffness could thereby be caused by qualitative or quantitative molecular changes; e.g., a formation of advanced glycation end products changing the properties of collagen[35]. An increase of endomysium, even if only relatively, likely has an effect on muscle stiffness. Increased muscle stiffness is further assumed to affect the contraction and relaxation processes of the entire muscle via impaired myofiber displacement[68]. Changes of muscle stiffness regarding age, gender, and exercise should be discussed in greater detail.

Mouse tibialis anterior muscle showed increased muscle stiffness with age. The authors thereby measured the stiffness of individual muscle fibers as well as muscle bundles and, in line with other studies, concluded that muscle stiffness is related to the ECM network. Muscle fibers themselves did not, however, show an increased stiffness in old age. As intramuscular tissue space remained unchanged and the analysis of individual IMCT components showed an increased amount of hydroxyproline as a marker for collagen[77], it can be assumed that the reported increase in muscle stiffness with age is due to a more densely packed collagen network in intramuscular connective tissue. Our finding of a relative increase in endomysium space during immobilization in humans would match the results found in mice. Bed rest is a model for aging as underscored by our concurring results. Increased muscle stiffness could hereby be influenced by the relative IMCT increase itself as well as by the described qualitative changes in the IMCT network. When analyzing human muscle biopsies of the vastus lateralis muscle, similar results were obtained as in the above cited mouse study. In summary, the proportion of connective tissue area in muscle increased with age, muscle showed increased passive stiffness, and passive stiffness could be attributed to connective tissue components in comparisons between muscle fibers and muscle bundles[78]. It can therefore be postulated that a relative increase in intramuscular connective tissue leads to increased muscle stiffness despite a lack of knowledge regarding underlying mechanisms.

Eby et al. evaluated passive muscle stiffness (shear modulus) through noninvasive shear-wave elastography in the biceps brachii muscle. They found increased muscle stiffness with age (upwards of 60 years) as well as increased stiffness in women compared to men at any age. Women's muscles generally tend to have a lower cross-sectional area and lower muscle strength than men's muscles. Passive joint torque is also higher in men compared to women. A potential relationship between passive joint torque and passive muscle stiffness might be tenuous. The authors suggested following up on these measurements with additional test subjects and then correlating shear-wave elastography with muscle biopsy results[79]. This would enable the comparison of noninvasive measurement results and histological results of

IMCT space and allow the analysis of individual IMCT components. As additional elucidation, gender differences can also be found in the tendon. Men here generally show a higher basal collagen synthesis rate as well as a more pronounced increase in collagen synthesis post exercise. This discrepancy is postulated to be an effect of higher estradiol levels in women as this hormone has been shown to correlate with reduced collagen synthesis and a higher rate of connective tissue injuries[80,81]. Though the tendon is generally less affected than muscle itself during immobilization and the responses of tendon and IMCT may differ[80], the divergence in IMCT synthesis rates between the sexes could analogously alter the stiffness of the respective atrophying muscles. Estradiol levels are further changed by the intake of contraceptive pills and differ between pre- and post-menopausal women. A special focus should therefore be placed on post-menopausal women in that the combination of reduced estradiol levels and generally lower levels of activity could additionally facilitate injury and hinder successful recovery. Gender differences should be investigated further as research could enhance knowledge about connective tissue injuries, their recovery, and potentially most importantly, their prevention.

Muscle fibers have long been known to adapt based on changing levels of physical activity. Reviews by Kjaer et al. as well as Mackey et al. demonstrate that intramuscular connective tissue and tendons dynamically adapt to disuse and physical activity. An increase in collagen synthesis were determined in both tissues after acute exercise[81,82]. This increase in collagen was primarily located in the perimysium[82]. In addition to an increase in collagen synthesis, an increase in protein degradation was also determined. Extended periods of exercise lead to increased collagen turnover and to an increase in the absolute amount of collagen. Kjaer et al. reported reduced stiffness in tendons with disuse, which was reversible by resistance exercise[82]. This is of interest in that increased collagen content, or increased intramuscular connective tissue content, is linked to increased muscle stiffness[33] and, based on the relative endomysium increase, we also hypothesize stiffness to increase during immobilization. A differential reaction in the muscle's connective tissue network compared to that of the tendon therefore appears to exist. Passive stretching is often integrated into exercise regimes or performed separately. Muscle groups show different responses to passive stretching, whereas passive muscle stiffness is reduced in the plantar flexors but not in the knee extensors[83]. Research on different training effects as well as stretching on muscle stiffness are, however, still scarce.

Increased stiffness is associated with functional impairments. Sarcopenia is not only characterized by loss of muscle mass and strength, but is also associated with increased collagen content and increased muscle stiffness[79]. Higher passive stiffness limits joint movements and the contractile ability of the muscle[34]. Transmission of shear forces is also presumably affected[27]. Muscle growth is furthermore regulated through muscle deformation. If

muscle stiffness increases, perceptions are likely to change and affect muscle growth and regeneration[34].

To summarize, immobilization and aging increase muscle stiffness, whereby the endomysium space increases relatively. Qualitative changes in the intramuscular connective tissue are postulated to influence muscle stiffness. Muscle stiffness hereby appears to be influenceable through exercise regimes.

5.3.4. Cross-linking and advanced glycation end products

We also stained biopsy samples for advanced glycation end products. These stains did not show any consistent results and could therefore not be properly evaluated. It is, however, still of interest to discuss possible implications of AGE increase in relation to stiffness. With an aging population, an increasing proportion of people suffer from diabetes and the consequences thereof. Even before this disease is diagnosed, increased blood sugar levels induce complications, such as retinopathy, nephropathy and neuropathy[84]. AGEs have been proposed to contribute to these complications[84].

AGEs are formed non-enzymatically through the Maillard reaction. Their formation is not only driven by glucose, but also via oxidative stress and other sugars, such as fructose. Multiple reversible reactions first occur. Their products include Amadori products such as glycated hemoglobin (HbA1c). These intermediate products then rearrange themselves into stable AGEs and form covalent cross-links[84,85]. Because the early steps of the Maillard reaction depend on the sugar concentration, it is natural that AGEs are found to be increased in diabetes[84]. The entire process takes several months and therefore long-lived tissues, such as connective tissue comprised of collagen, are more affected [85]. Formed cross-links impact tissue stiffness and therefore function[84].

Depending on their location, AGEs have been postulated to enhance stiffness, stimulate proinflammatory pathways, and further atherosclerosis[85]. They have been found to affect tissue remodeling, loss of elasticity, vascular permeability, and endothelial dysfunction through collagen-cross-links[84]. AGEs are further believed to influence a variety of diseases, not only diabetes, but also connective tissue diseases, rheumatoid arthritis, and renal disease[84]. Prevention is essential because AGE cross-links cannot easily be hydrolyzed[84]. It has hereby been suggested that the rate of AGE accumulation might be of greater importance than the absolute amount of AGEs[84].

As reviewed by Kragstrup et al., collagen cross-linking is known to increase with age and reduce with exercise in animals[66]. Mouse tibialis anterior muscle showed triple the amount of AGEs in old mice compared to adults[77]. If this is indeed also the case in humans and cross-links do affect muscle stiffness, this finding further stresses the importance of exercise training throughout life, but especially in the elderly. These findings have to date not been replicated in

humans, although this could be a result of study designs and the muscle studied. It has been shown that advanced glycation end products do accumulate with age in humans in that the proteins are exposed to more glucose over the course of a longer life span. Taken together, both forms of cross-linking (collagen and advanced glycation end products) could impact force generation in muscle[66].

Staining intensity has often been used to quantify AGE concentrations. No general method has to date been established, which renders a comparison of the results in different studies difficult. The most commonly used methods include immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and high performance liquid chromatography (HPLC)[84].

We used immunohistochemistry and failed to observe any AGEs in our stains. Assuming that this result is correct and that no detectable AGEs were in fact present (as opposed to the assumption of flaws in the staining protocol), the examined population might be too young and too healthy to histologically determine a significant amount of AGE cross-links. It is also possible that the elapsed time period of 55 days between the two measurements was not long enough to detect any changes histologically. These stains should therefore be repeated on a larger and more diverse study group.

In summary, advanced glycation end products accumulate with aging and are thought to affect a variety of tissues, pathways, and diseases, e.g., AGEs are postulated to enhance muscle stiffness through covalent cross-links.

5.3.5. Aging

As set forth above, muscles undergo various changes with aging. These changes include, but are not limited to, muscle atrophy and enhanced muscle stiffness. This section will elaborate on intramuscular connective tissue changes during aging.

Alnaqueeb et al. observed that rat muscle changes in aging are a combination of decreasing muscle fiber size and number[64]. They further determined an endomysium increase early in postnatal development and again in senility, between which endomysium remained stable[86]. Based on these results, multiple animal studies have histologically observed increasing collagen content as well as a biochemically determined increasing collagen concentration in the ECM of muscles[66].

These findings are in line with our results as the histological analysis of collagen content was predominantly determined as collagen area (IMCT area) in relation to muscle cross-sectional area. Collagen concentration was biochemically measured as hydroxyproline per muscle weight[66]. A relative increase in intramuscular connective tissue would show an increase in both of these parameters. Some studies in humans have demonstrated divergent results with no change in collagen content with age[66]. Kragstrup et al. discuss that these divergent results could result from different studied muscle parts so that different IMCT layers were included in

the study. Animal studies mostly use whole muscle while human studies rely on muscle biopsies, which naturally include less epimysium and perimysium[66].

Aging has also been related to increasing muscle stiffness and changed ECM structure in both animals and humans[35,68]. While stiffness generally was discussed above, within the context of aging, stiffness has been found to increase with age, which increase is attributable to the intramuscular connective tissue[35]. Wood et al. found double the amount of hydroxyproline in old mouse tibialis anterior muscle compared to younger adult mice. They postulated that, because connective tissue space remains constant, the connective tissue network becomes denser and therefore stiffer[77]. As discussed above, increased stiffness could be an effect of a relative enrichment in IMCT per muscle as well as the presence of more densely packed and cross-linked collagen fibers. If intramuscular connective tissue does accumulate relative to muscle fiber, questions of functional implications and suggestibility of IMCT metabolism arise. As discussed, an increase in intramuscular connective tissue with age is closely related to enhanced muscle stiffness and potential functional changes.

5.3.6. Artificial gravity

Artificial gravity has been postulated as an integrated countermeasure to physiological adaptations to spaceflight[2]. The object of the AGBRESA study was to determine the countermeasure effectiveness of artificial gravity via a human short-arm centrifuge[18]. While no significant effect of artificial gravity on intramuscular connective tissue was observed in our study, other AG findings, as well as potential future applications, will be discussed below.

Previous studies have examined artificial gravity as a countermeasure to muscle atrophy. A 21-day bed rest study found a decreased cross-sectional area in the soleus muscle in the control group but no such change in the AG intervention group. Subjects in the intervention group were subjected to 1 hour of AG (2.5 g at the feet) per day and were allowed to make use of the muscle pump as the subjects themselves deemed necessary. The authors observed that AG could prevent some effects of microgravity, in particular muscle volume and strength[87]. As described in other bed rest studies, AGBRESA subjects were allowed to use the muscle pump at their own discretion. While our study did not measure any functional parameters, such as strength, we did examine muscle atrophy expressed as fiber cross-sectional area. Contrary to previous findings, we did not observe any significant effect of artificial gravity on the soleus muscle although muscle atrophy was more pronounced in the control group compared to the intervention group. This discrepancy of results could be due to diverging gravitational forces. The described 21-day bed rest study used 2.5 g (measured at the feet), while the AGBRESA study sought to induce forces of 1 g at the center of mass. Gravitational forces in the lower limbs were therefore lower in the AGBRESA subjects, and this could contribute to a weaker protective effect against muscular deconditioning.

Edmonds et al. have additionally proposed the combination of intermittent centrifugation and exercise as a comprehensive countermeasure. Subjects underwent daily exercise on the centrifuge and showed slight fitness improvements[88]. It would therefore be of interest to examine bed rest subjects undergoing different exercise regimes, in particular resistance exercise, on a short-arm centrifuge. Because Rittweger et al. concluded that AG was unlikely to counteract muscle or bone metabolic changes in a 5-day head-down tilt bed rest study[89], and because exercise regimes during spaceflight have to date not proven sufficiently effective[17], a combination of artificial gravity and exercise appears to be the logical sequence. In summary, artificial gravity has been shown to have effects on muscle atrophy although the present study did not observe any effects in its bed rest subjects. Additional research on future applications of AG should therefore focus on the combination of artificial gravity and exercise regimes.

5.4. Clinical implications

Our findings demonstrated a relative increase in intramuscular connective tissue during long-term immobilization. As described above, while immobilization is relevant in spaceflight, it is even more relevant for the elderly and for those hospitalized. Fibrosis has furthermore been described in multiple diseases, including neuromuscular disorders[37]. Increase of the endomysial space has been described in aging as well as in spastic muscles[27]. Intramuscular tissue in general is additionally associated with overloading injuries[27]. It is therefore essential to discuss possible clinical implications of intramuscular connective tissue changes.

Increased collagen content located in the intramuscular connective tissue has been found to enhance muscle stiffness[28,33]. If increased endomysium content does in fact lead to increased muscle stiffness, this could have substantial functional implications for muscle contraction as discussed above but which nevertheless requires further examination.

Consequences for the elderly are of particular importance and are discussed above. Not only muscles, but also bones, are altered with aging. Muscle stiffness occurs although the exact role increased muscle stiffness plays in aging is not yet clear. It could have a protective effect in preventing overstretching and stabilizing atrophying muscle, it could also hinder muscle contraction and reduce the range of motion at the joint[77]. The potential interaction between sarcopenia and osteopenia would lead to increased frailty and risk of injury as well as impaired regeneration. Understanding the mechanisms could improve prevention and treatment plans and therefore provide a better quality of life.

Influences on collagen synthesis by disuse and training would also have implications for training and rehabilitation regimes[82] in that training units for athletes, as well as exercises for patients, should be planned for maximal benefit and minimal injury risk. Too dense or intense training sessions could lead to an increased risk of injury absent the benefit of increased

collagen synthesis. Actual increase in collagen content in tendons only occurs after extended exercise periods and after a time period for collagen reconstruction[82]. This latency in absolute collagen increase could leave a window with a higher risk of injury.

Increased fibrosis has been described in multiple diseases[27], e.g., contractured muscles from children with cerebral palsy are significantly stiffer and have a higher collagen content[33]. This finding relates to the ECM as individual muscle fiber stiffness was not increased in the contractured muscles although the fibers were smaller[63]. This means that in contractured muscle, increased stiffness and a reduced range of motion is related to changes occurring in the extracellular matrix space and not the muscle fibers themselves. Many neuromuscular disorders also result in muscle contractures, and it can be assumed that these muscles show similar characteristics to muscles found in patients with cerebral palsy. The question of how to treat contractured muscle and thus the altered intramuscular connective tissue must yet be determined. Cerebral palsy is currently treated on the muscular level, whereas the underlying injury is located in the brain[90]. As muscles react differentially to stimuli, it is essential for potential therapeutic approaches to study the muscles and various diseases individually.

In summary, determining the underlying pathways that regulate intramuscular connective tissue are significant for developing therapeutical approaches for multiple disorders as well as for establishing preventive measures, e.g., effective exercise regimes, with a particular focus on interventions for complications and injuries in the elderly.

5.5. Limitations

Bed rest studies are complex and require extensive planning despite subjects being rigorously selected via physical and psychological criteria. Many research teams are involved in conducting bed rest studies and each individual team pursues their own study objectives. These individual objectives do not necessarily correlate with the general study objective, which in AGBRESA was determining the countermeasure effectiveness of artificial gravity. Our primary goal was, however, to examine intramuscular connective tissue during long-term immobilization and the AGBRESA study provided a unique setting to do so.

A natural limitation of bed rest studies is the limited number of subjects. The ABGRESA bed rest study included 24 subjects. Biopsies could not be acquired from all subjects, so that a total of 21 subjects were included in the present study. Though a small number of test subjects is generally less representative of the general population and reduces the external validity of results, astronauts are selected through an even more rigorous process. Ethical considerations and limited resources for conducting long-duration bed rest studies must also be taken into account. Under these considerations, we deem our number of subjects to be sufficient for conclusive results.

Bed rest subjects also tend to be younger than active astronauts, which represented the modeled population group in our study. No conflict here existed in that our object was to examine IMCT during long-term immobilization. As muscle atrophy can be increasingly found in the elderly, no significant effects of age discrepancy were expected within our relatively young subject group.

In AGBRESA, subjects were pseudo-randomly assigned to the control or to one of two intervention groups while balancing the genders in the individual groups. The observer was blinded to time and group allocation when analyzing the images. This ensured the least possible bias during the analysis process.

A possible limitation of biopsy studies is that the sample taken is not representative of the muscle structure as a whole and that inter-individual differences influence the results. Our results are consistent throughout our subjects and we included a minimum of 50 muscle fibers in each analyzed stain. Our results also affirm our hypothesis based on previous research, so that we believe our data to be representative.

Our study specifically assessed the endomysium area, but did not examine the individual components of the intramuscular connective tissue, so that we cannot comment on the composition or structure of endomysial changes. This aspect should be assessed in further studies.

We deem our study and results to be valid. Additional studies can reduce limitations based on these findings. Details of further study questions will be detailed below.

5.6. Outlook

Many studies have examined muscle changes during disuse although a high proportion have focused on active contractile elements and not on intramuscular connective tissue. New interesting research questions arise based on our findings. While these have in part already been touched upon in the above sections, they will be further outlined below.

Because we only determined the histological change of the endomysium area, it would be of further interest to correlate these findings with measurements of stiffness as well as with histological stains of individual endomysial components. Stains should include different collagens, in particular collagens I and III, as well as elastin. Endomysial collagens are primarily produced by fibroblasts[81]. Fibroblasts are located in the endomysial and perimysial connective tissue and are linked to the intramuscular connective tissue network so that they can sense changes in mechanical loading[81]. It would therefore be of interest to additionally examine fibroblasts regarding their quantity as well as their activity during different loading conditions (immobilization and post-exercise).

Stiffness is further implied to impair muscle function. To examine this hypothesis, subjects should not only receive muscle biopsies, but should also undergo functional measurements (muscle strength) on the day of the biopsy in order to correlate both results.

Because we examined a healthy group undergoing voluntary bed rest, further immobilization or disuse studies should include different populations to the extent ethically feasible. Studies should include comparisons of younger and older age groups, genders, as well as preexisting connective tissue and muscle disorders. Focusing on the elderly is of particular importance as bed rest is an accelerated model for aging.

Muscles show different responses to immobilization and muscle fibers atrophy differentially. While it is probable that the quantitative reaction of intramuscular connective tissue in different muscles remains the same, it would be of interest to verify this assumption. A simultaneous qualitative analysis in different muscles would also be of interest.

It must be further assumed that nourishment of muscle fibers depends on capillary density and blood flow which is in turn influenced by diffusion length. Stains should therefore examine the number of capillaries during immobilization and assess how they could influence nourishment of the atrophied muscle fibers and influence function via this pathway.

In addition to determining the underlying mechanisms relating to intramuscular connective tissue and the corresponding functional impairments, it is of particular importance to determine countermeasures. We did not find any effects of artificial gravity. Future studies should therefore focus on training regimes which could ideally be incorporated into physical therapy or rehabilitation, or the combination of training regimes with artificial gravity regarding potential long-duration spaceflights. As conditions such as cerebral palsy impair children at a very young age, analysis of biopsy samples from young children could enhance the understanding of regulatory mechanisms and their changes with aging.

In summary, studies should focus on a more qualitative approach after having determined the quantitative endomysium content in different populations and different circumstances.

5.7. Conclusions

We examined the endomysium content of the soleus muscle during 55 days of bed rest through the endomysium-to-fiber-area ratio and the endomysium-to-fiber-number ratio. As endomysium-to-fiber-area increased and endomysium-to-fiber-number remained unchanged, a relative endomysium increase was observed.

Enhanced intramuscular connective tissue content has been postulated to increase muscle stiffness which in turn could have functional implications for muscle contraction.

Intramuscular connective tissue changes are not only relevant in disuse conditions but also during the physiological aging process. The search for countermeasures is crucial as humans strive to explore space and populations grow increasingly older. No effective integrated

countermeasure has to date been found. The option of artificial gravity has been explored in the AGBRESA study but requires additional research, as do exercise regimes or the combination of both countermeasures.

As well as influencing physiological conditions, intramuscular connective tissue also plays a role in various disorders. Additional research is required to understand the underlying mechanisms and to develop new therapeutic and preventive approaches.

In conclusion, our findings show connective tissue changes during immobilization in humans and are therefore a basis for additional research.

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7. Appendix

7.1. List of figures

8. Prepublication of results

Presentations

December 7, 2019: 4th Human Physiology Workshop, Cologne

March 6-7, 2020: Second KNIMS Annual Conference (Kompetenznetzwerk Immobilisationsbedingte Muskelstörungen - Network of Expertise for Immobilization-induced Muscle Disorders), Cologne

Poster Presentations

January 27-30, 2020: Human Research Program Investigators' Workshop, Galveston, TX

September 30-October 2, 2021: 100th Meeting of the German Physiological Society, Frankfurt

Publication

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