Diplomarbeit

Does exercise during immobilization prevent muscle loss?

eingereicht von

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zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

Institut für Physiologie

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Graz, den 08.10.2014

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Graz, am 08.10.2014 David Manghabati

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Abstract

Introduction: Immobilization is often seen in diseases and is accompanied with a decline of skeletal muscle mass and strength. The underlying molecular mechanisms are yet not fully understood. This diploma thesis gives an overview of the pathophysiology of skeletal muscle and the effects of immobilization and exercise on the skeletal muscle. Using OMIC technologies, data collected during an ESA bedrest were explored with focus on how exercise effects the molecular mechanism of muscular atrophy. Compared were data of bedrest immobilization with and without exercise intervention. The results have applications in cardiology, geriatrics and immobilization.

Aims and Objectives: The aim of this diploma thesis is to explore how bed rest affects the muscular system of the human body, with emphasis on the underlying molecular mechanisms and if any differences can be observed depending on the type of intervention. We tested the hypothesis that exercise prevents muscle loss during bedrest immobilization and that this will be seen in the observed molecular changes.

Methodology: Twelve healthy male volunteers participated in this 21-day six-degree head down bed rest ESA study at MEDES, Toulouse. Their average weight was 69.8 $\pm$ 8 kg, their average age was 34 $\pm$ 8 years and they had an average height of 1.76 $\pm$ 6 cm. Blood samples taken pre- and post-bedrest where used to examine changes in the gene expression important for skeletal muscle function.

Results: Eight genes KCNH2, FBXO32, IGF1, YAP1, PRSS12, SGCB, NOS1 and TRIM63 involved in muscle physiology had expression values changes with a fold change greater than +1.5 or less than -1.5. KCNH2, NOS1 and GDAP1 (down regulated) and TRIM63 and HSPB3 (up regulated) are expressed genes that are in common between the bedrest and bedrest and exercise group.

Conclusions: Bedrest has an effect on gene expression. Transcriptomics can detect these changes. No gene was found that has significantly changed between the group *bedrest* and *bedrest and exercise*.

Abstract in German

Einleitung: Krankheiten gehen oft mit Immobilisierung und einem Verlust von Muskelmasse einher. Die zugrundeliegenden molekularen Mechanismen sind bisher nicht geklärt. Diese Diplomarbeit gibt einen Überblick über den aktuellen Wissenstand der Muskelphysiologie und den Auswirkungen von Immobilisation und sportlicher Betätigung auf den Skelettmuskel. Die Daten einer ESA Studie werden daraufhin untersucht, ob sportliche Betätigung einen Einfluss auf die molekularen Mechanismen beim Muskelabbau hat. Die Ergebnisse werden mit Daten einer Diplomarbeit von Tristan Jaskolla verglichen, die die zugrundeliegenden Ursachen des Muskelverlustes untersucht hat. Unsere Ergebnisse können praktische Anwendung in der Kardiologie, Geriatrie und bei Immobilisation finden.

Zielsetzungen: In dieser Diplomarbeit wird die Frage untersucht ob Bettlägerigkeit das muskuloskelettale System des menschlichen Körpers beeinflusst. Das besondere Augenmerk liegt hier auf den zugrundeliegenden molekularen Ursachen. Des Weiteren wird der Einfluss von sportlicher Betätigung auf die molekularen Mechanismen und den immobilisations-aassoziierten Muskelabbau untersucht.

Methoden: In dieser von der ESA in MEDES, Toulouse durchgeführten Studie partizipierten zwölf gesunde männliche Teilnehmer. Die Probanden lagen während dieser 21-tägigen Studie mit sechs Grad gesenktem Kopfende im Bett. Sie hatten ein durchschnittliches Gewicht von 69.8 $\pm$ 8 kg, ihr durchschnittliches Alter war 34 $\pm$ 8 Jahre und ihre durchschnittliche Größe war 1.76 $\pm$ 6 cm. Blutproben der Patienten wurden auf eine Veränderung der Regulation relevanter Gene hin untersucht.

Ergebnisse: Acht Gene KCNH2, FBXO32, IGF1, YAP1, PRSS12, SGCB, NOS1 und TRIM63 mit einem Fold change von $\geq +1.5$ oder ≤ -1.5, wurden als muskelphysiologisch relevant erkannt. Die herunterregulierten Gene KCNH2, NOS1 und GDAP1 und die heraufregulierten Gene TRIM63 und HSPB3 (wurden herauf reguliert) wurden in den beiden Studiengruppen *Bettlägerigkeit* und *Bettlägerigkeit mit Sport* exprimiert.

Schlussfolgerungen: Bettlägerigkeit beeinflusst die Genexpression. Mit Transcriptomics können diese Veränderungen beobachtet werden. Zwischen den Studiengruppen Bettlägerigkeit

und Bettlägerigkeit mit körperlicher Aktivität hat sich die Genexpression der untersuchten Gene nicht signifikant verändert.

1 Introduction

Many diseases such as cancer, AIDS, COPD and injuries frequently come with immobilization and are often accompanied by the side effect of muscle loss by mechanical unloading of the muscle. Many studies have been carried out focusing on the morphological changes. We want to go one step further, by using OMIC technologies. Instead of studying isolated biological molecules, we will analyze large sets of molecules. By analyzing cDNA we want to explore the connection between bedrest and adaption/response of human skeletal muscle; to investigate the underlying complex molecular organization; to scrutinize biological pathway dynamics at work and to develop new countermeasures. The focus of this diploma thesis is on the skeletal muscle, how the muscle acts and reacts, how the muscle function and structure is affected by bedrest and immobilization. We hope that our findings can be applied in medical fields such as geriatrics, cardiology and immobilization.

1.1 Muscle

1.1.1 Muscle Anatomy

The human body has three types of muscle cells, cardiac, smooth and skeletal (striated) muscle. Most skeletal muscles are attached to two bones via tendons across a joint to allow the movement of body parts. Tendons are a dense connective tissue connecting the muscle to the bones. A muscle is enwrapped by a layer of fibrous tissue, the fascia. The function of skeletal muscle is the movement of body parts by contraction. It is controlled by the somatic nervous system.

1.1.2 Muscle Physiology

Striated muscle cells develop from mononuclear myoblasts. They fuse into polynuclear myotubes and differentiate into muscle fibers. Every muscle fiber is made out of a bunch of myofibrils with a diameter of one micrometer, which cross the whole length of a muscle fiber. Myofibrils are composed of repeated sequences of actin and myosin. Actin and myosin form the smallest morphological and functional unit of the muscle fiber, called sarcomere. Under the

microscope, the skeletal muscle fiber shows a characteristic striation due to the periodic pattern of thin (actin) and thick (myosin) myofibrils (Figure 1) (1).

The plasma membrane in muscle fibers is called sarcolemma. The flattened myonuclei are pressed against the inside wall. In the cytoplasm, here referred to as the sarcoplasm, myofibrils containing myofilaments can be found. The smooth endoplasmatic reticulum in muscle cells is called sarcoplasmic reticulum. It surrounds the myofibrils and holds stores calcium (Ca) ions needed to cause muscle contraction. The sarcomere has a length of 2.2 to 2.4 micrometers and is defined as a segment between two neighboring Z-Lines. Z-lines appear as dark lines in electron microscopy of cross-striated muscle. The surrounding Z-line is the I-band; it manly consists of thin (actin) and titin filaments. Next to the I-band lies the A-band. It contains the whole length of a thick (myosin) filament. The part in the A-bands without thin filaments is called H-zone. The H-zone is divided by the M-line in the middle. Myosin filaments are linked to the M-line via structural proteins.

The proteins are located on the sarcomere as following:

- actin (thin filaments) is located in the I-band and in the A-band until the beginning of the H-zone
- myosin (tick filaments), located in the A-band, is cross-linked at the M-line to structural proteins, e.g. myomesin
- titin is a large sized protein with a length of more than one micrometer. Titin reaches from the Z-disc to the M-line, is connected to myosin and contributes to muscle assembly and resting tension
- actin and titin are cross-linked at the Z-disc via alpha-actinin

One myosin filament contains 300 myosin units. Every molecule is made out of two heavy chains and four light myosin chains. The heavy chains are composed of one α-helical tail domain and one globular head domain. The tail domain consists of two heavy chains wound around each other forming the neck- and tail domain. The distal part of the head domain has a catalytic function. Actin binds here and can hydrolyze adenosine triphosphate (ATP). The proximal part of the head domain with its two light chains operates as a lever during contraction.

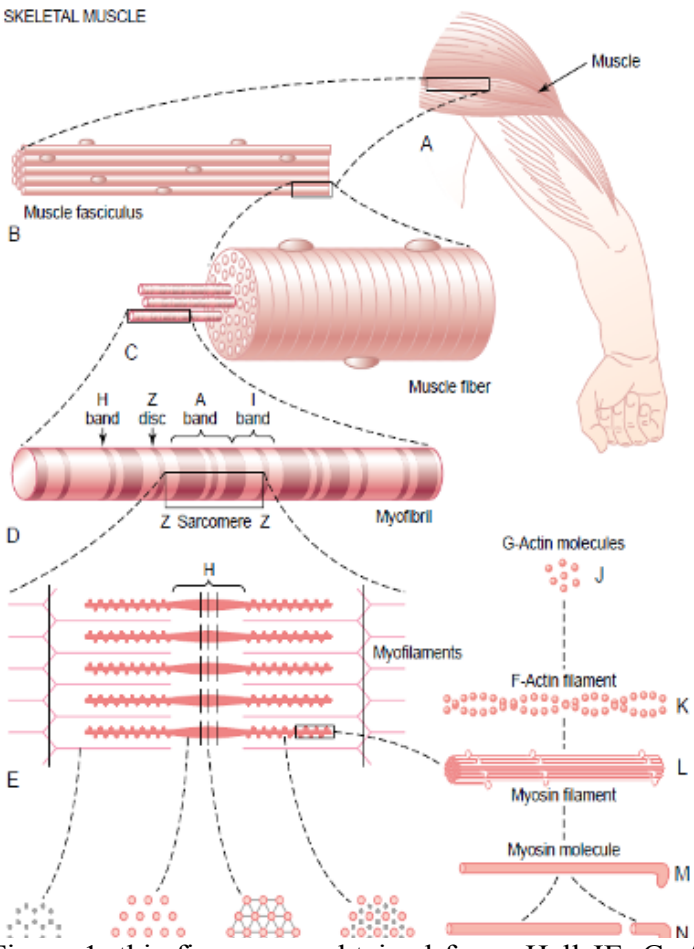

Figure 1, this figure was obtained from: Hall JE, G. A. (2006). Textbook of Medical Physiology (p. 1152). St. Louis: Elsevier Saunders. Figure 1 gives an overview of the skeletal muscle structure.

The actin filament is composed of three protein components: filamentous (F)-actin, tropomyosin and troponin F-actin is a single stranded left-handed helix with a diameter of ten nanometers. It is formed by globular (G)-actin monomers, which binds ATP. It is assumed that the actin-myosin interaction causes muscle contraction. Tropomyosin molecules are wrapped around F-actin to prevent muscular action. It binds to the actin's active sites during rest and inhibits the interaction between actin- and myosin filaments. There are three different troponin molecules building complexes which play a role in muscle contraction. Troponin I binds to actin, holds the actin-myosin filaments in place and thus inhibits muscle contraction. Troponin T binds to tropomyosin and helps to position it on actin. Troponin C has four EF-hands to bind Ca (2). The binding of calcium leads to a conformational change, such that Troponin I dislocates and the muscle can contract.

1.1.2.1 Neuromuscular transmission

All muscle fibers innervated by one motor neuron form the motor unit (3). Motor neuron endings and the muscle fiber build the neuromuscular junction. The neuromuscular junction transfers impulses from the nerve endings to the skeletal muscle fibers. The motor end plate consists of the branching nerve terminals invaginating into the muscle fiber surface (1). Although being invaginated the nerves have no direct contact to the muscle fiber plasma membrane. The motor end plate is covered by one or more Schwann cells shielding it from the surrounding tissue. The space in-between is the synaptic cleft. An action potential arriving at the nerve ending (presynaptic axon) opens membrane located, voltage controlled calcium channels. Calcium diffuses into the synapse inducing a fusion of acetylcholine (ACh) filled vesicles with the axon membrane. ACh is released by exocytosis into the synaptic cleft and diffuses to the nicotinic acetylcholine receptor, a ligand-gated ion channel, in the postjunctional fold. When ACh binds, the receptor opens, mainly leading to a sodium ion influx. This creates an excitatory postsynaptic potential called end-plate potential.

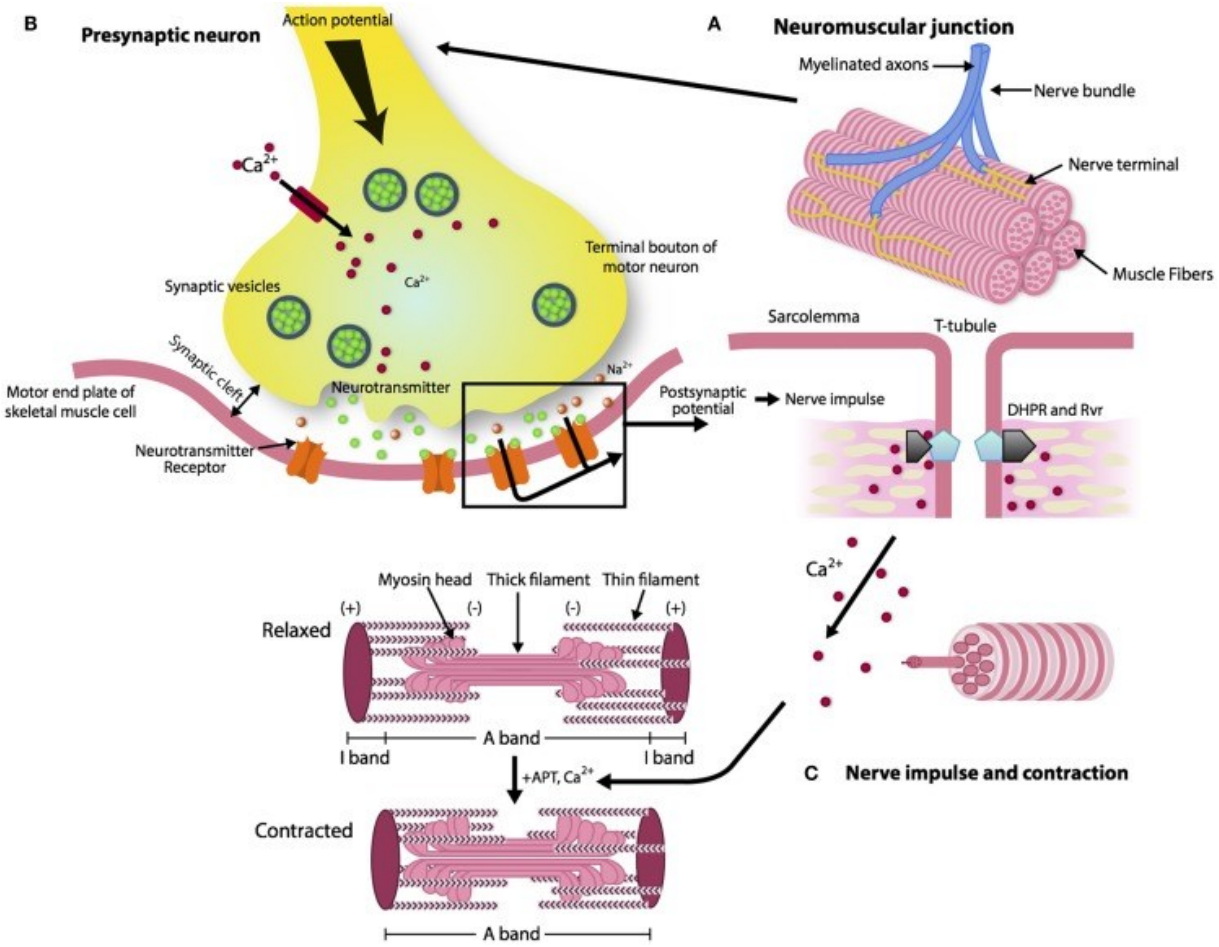

Figure 2 (A,B) The motor nerve terminal, the synaptic basal lamina and the muscle fiber and muscle membrane form the neuromuscular junction. An action potential reaching the end of a motor nerve opens Ca channels and Ca influxes into the neuron leading to Ach release into the synaptic cleft. (C) In the sarcolemma located dihydropyridin receptors become activated by AchR. Also ryanodine receptors are induces by AChR. Intrasarcoplasmic reticulum located Ca influxes via the ryanodine receptor and binds to troponin C. The cross-bridge cycle can start now.

This figure was obtained from: Gonzalez-Freire M, de Cabo R, Studenski SA, Ferrucci L. The Neuromuscular Junction: Aging at the Crossroad between Nerves and Muscle. Front Aging Neuroscience

1.1.2.2 Excitation-contraction coupling

The process between an action potential reaching the sarcolemma and activation of the cross-bridge cycle is called excitation-contraction (E-C) coupling.

The transverse tubule-sarcoplasmic reticulum system is invaginated sarcolemma, very small and runs transverse to the myofibrils. It crosses completely through the muscle fiber from one side to the opposite side. The point from where they originate from the cell membrane they are open, i.e. the transverse tubule-sarcoplasmic reticulum system communicates and contains extracellular fluid. When an action potential reaches the muscle fiber membrane, it leads to a potential change in the T-tube and causes muscle contraction.

The sarcoplasmic reticulum has two major components: longitudinal tubules and terminal cisternae. It is an intracellular membrane system storing high concentrations of calcium ions. Longitudinal tubules surround all surfaces of contracting myofibrils, terminal cisternae's are about T-tubules. One transversal tubule and two terminal cisternae build a triad. A triad is the location where the E-C coupling takes place. On the T-tubule side of the triad, voltage controlled calcium channels called dihydropyridin receptor (DHPR) are located in the membrane. DHPRs are in contact with other specialized calcium channels called ryanodine receptors, located in the membrane of the terminal cisternae. An action potential spreading in the T-tubule induces a molecular rearrangement of the DHPR. As a result ryanodine receptors release calcium ions. Calcium binds to troponin C and the cross bridge-cycle starts.

1.1.2.3 Molecular mechanism of muscle contraction

The molecular mechanisms of muscle contraction are based on the interaction between actin and myosin, also called cross bridge-cycle. The steps are:

1. ATP binds to the myosin head and releases the myosin from the actin filaments.
2. ATPase activity of the myosin head cleaves ATP into adenosine diphosphate (ADP) and phosphate (P). This induces a conformational change of the myosin head, which moves towards the Z-line and binds to a new actin monomer. To be able to bind, first the troponin-tropomyosin complex has to bind calcium ions; that uncovers the myosin binding sites of actin and myosin binds

3. The bonding of the head of the cross-bridge and the active side of actin leads to a conformational change. The head tilts towards the neck and pulls the actin filament by about 6-8 nanometers. The power needed has been provided by the ATP hydrolysis in step 2.
4. ADP dissociates from the head, leading to a further tilting (2-4 nanometers)
5. After the dissociation of ADP the head is highly bound to actin. On normal physiological circumstances ATP binds to the head and the cycle can start again.

Isotone and isometric contraction

There are two types of contraction: isotone and isometric contraction. In isotone contraction actin and myosin shift against each other around ten nanometers per cycle. Actin is pulled between the myosin filaments, which shortens the muscle.

In isometric contraction, the muscle ends are fixed so that the actin binding site, seen relatively to the myosin head is fixed. This means that Step 3 and 4 are hindered. Instead, the myosin head undergoes an elastic deformation in the region where the lever arm is fixed in the catalytic domain. This leads to a reset force and to active muscle strength.

1.1.2.4 Muscle fiber types

One way to classify muscle fibers is by the primary type of the myosin heavy chain (MHC). In mammalian skeletal muscle, initially three types of motor units have been identified: slow, fast fatigable and fast fatigue resistance motor units consisting of type I, IIa and IIb fibers (3). In rats, a fourth motor unit consisting of type IIx fibers has been detected (4). There are four different MHC gene encoded proteins in striated muscle: slow type I MHC (coded by MYH7 gene) and three fast MHC types: IIa (coded by MYH2), IIx (coded by MYH1) and IIb (coded by MYH4) (5). The types primarily expressed in a given motor unit determine the intrinsic contraction speed. Type I and IIa fibers are more oxidative and type IIb fibers are more glycolytic. In human muscles there are only type I, IIa and IIx fibers expressed (6). These fibers generate almost the same force per unit cross-sectional area, but their individual maximum shortening velocity (type IIx is approximately four to five times faster than type I) and their

capacity generating power differ (7). Additionally, there are intermediate hybrid fibers, which are composed of type I and IIa, or IIa and IIx, or IIx and IIb MHCs, which are also normally seen in muscles (8). They become more when there is a fiber shift due to various reasons like exercise, electrical stimulation, muscle atrophy or bed-rest.(6)

The differences between fast and slow fibers are:

- Fast fibers are twice as large as slow fibers
- Fast fibers have a two to three times higher concentration of enzymes preferring anaerobic metabolism for rapid release of energy like high resting phosphocreatine, phosphorylase and phosphofructokinase from the phosphagen and glycogen-lactic acid energy systems (9) This leads to maximum power development for a short period of time (seconds) (1) due to consumption of energy or gathering of metabolites (10).
- Slow fibers are specialized in generating aerobic energy. Therefore they have much more active enzymes for metabolic energy generation. Furthermore, the number of mitochondria and myoglobin is much higher than in fast fibers. Myoglobin is a hemoglobin like protein that increases oxygen diffusion rate in fibers transferring one oxygen molecule from one myoglobin to another (11).
- Slow twitch fibers are surrounded by more capillaries than fast twitch fibers

In summary, fast twitch fibers generate max. power for seconds to a minute and slow twitch fibers provide strength for contractions over minutes to hours (1).

The combination of the fast types and the function of the different motor units can be seen in figure 3.

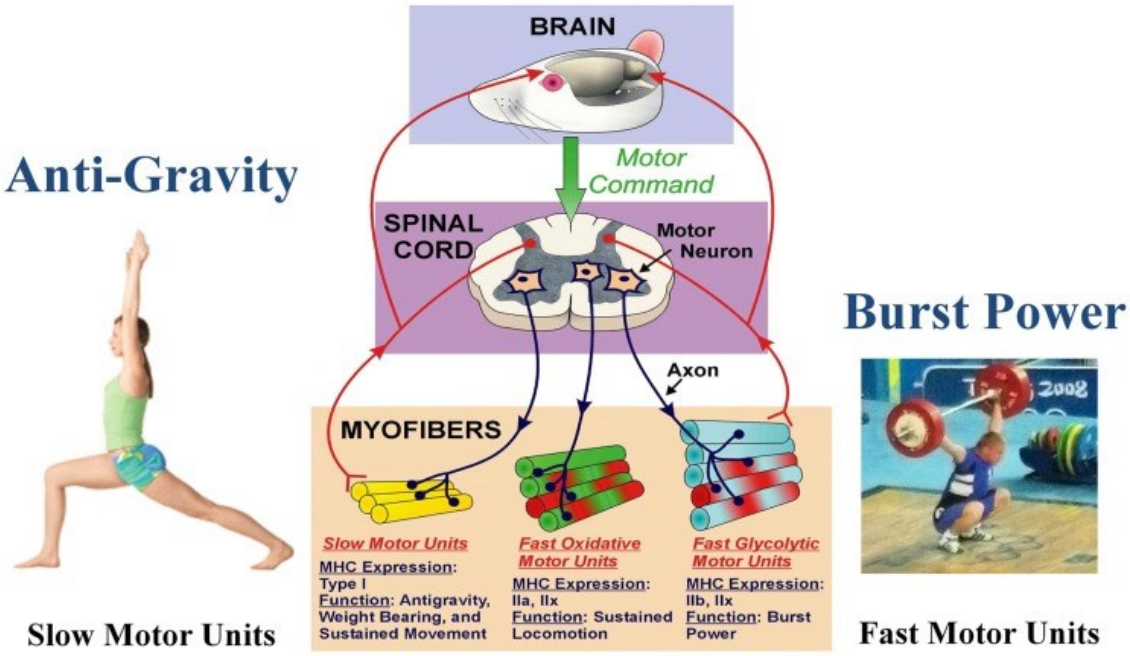

Figure 3 The brain gives the command to the motor neuron to perform a movement. The motor neuron in turn stimulates muscle fibers to contract. All muscle fibers innervated by one motor neuron form the motor unit. One motor unit consists of the branching nerve terminals of a single motor neuron invaginating into the muscle fiber surface. Depending on it function each motor unit can express different MHC phenotypes. Every single myofiber has the ability to express a single MHC isoform, or a mix of them. IIb and IIx are fast twitch fibers generating maximum power for seconds to a minute and I is a slow twitch fiber providing strength for contractions over minutes to hours

This figure was obtained from: Baldwin, K. M., Haddad, F., Pandorf, C. E., Roy, R. R., & Edgerton, V. R. (2013). Alterations in muscle mass and contractile phenotype in response to unloading models: role of transcriptional/pretranslational mechanisms. Frontiers in Physiology, 4, 284. doi:10.3389/fphys.2013.00284

1.1.2.5 Muscle pump hypothesis

The muscle pump developed after observations by Pollack and Wood who demonstrated that there is a relationship of gravity and walking on the intravasal venous pressure in human limbs; when standing on toes the calf muscle contracts and venous pressure falls at the ankle (12).

Folkow et al showed that when human calf muscles contract, the flow of blood is higher when the limbs are in a standing position and lower when the limbs are in a supine position (13). In upright standing humans, 70% of the circulating blood is below the heart and is mostly found in the venous vessels (14). Therefore the skeletal muscle pump plays an important role in coordinating local and systemic blood flow during locomotion (please see figure four). A singe muscle contraction can mobilize up to 40% of the intramuscular located blood and muscle contraction is an important way to centralize peripheral located blood by compressing the venous vessels (15). If venous valves are working normally, the muscle pump provides important support during exercise. First, rhythmic muscle contraction and relaxation provides a big amount of energy needed for peripheral blood flow circulation, it might also provide most of the energy needed to return blood from the limbs back to the heart (16). In addition, rhythmic contraction provides 30-60% of the energy needed for the muscular blood flow (16,17). Furthermore, when the muscle contracts concentrically, blood is pressed from the distal parts of the body back to the heart, thereby increasing venous return, stroke volume and cardiac output which is needed during exercise (18). For example contracting calf muscles raise intraveinal pressure up to 140 mmHg and drives venous blood from the deep lower leg veins into the popliteal and femoral vein (19).

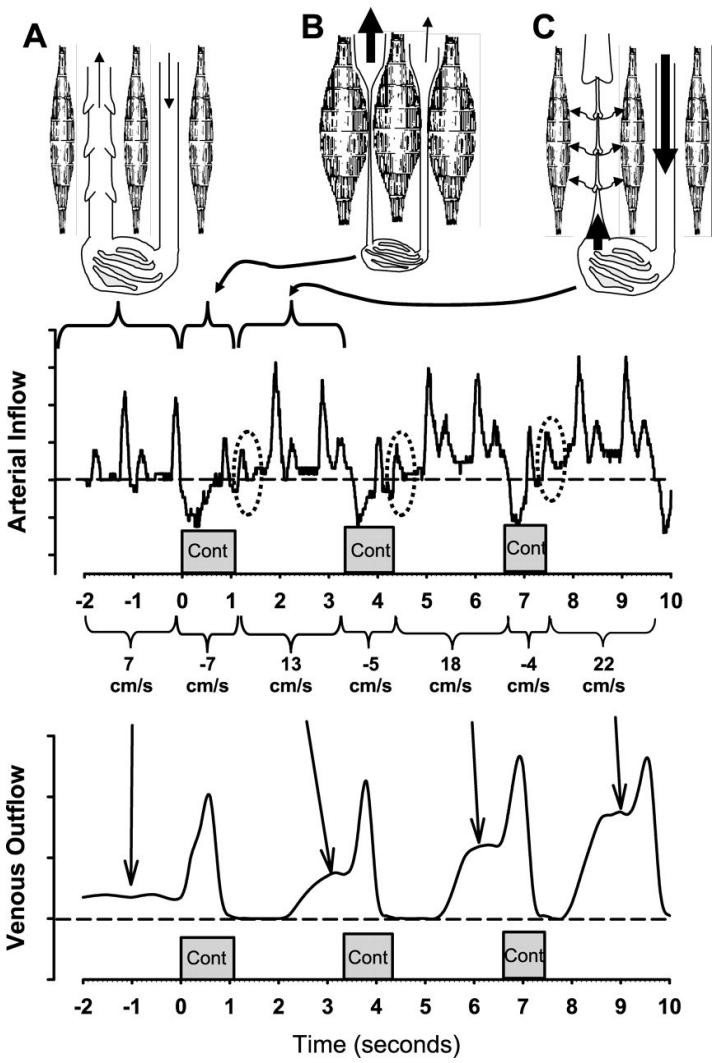

Figure 4

Rhythmic forearm handgrip exercise with contraction periods (Cont in shaded boxes). At brachial artery arterial inflow is measured. Venous return is the sum of all veins taking blood back from the forearm. Arterial outflow figure shows negative arterial inflow during contraction and short increase of inflow after releasing (dotted circle). Venous outflow is adapted schematically from data by Anrep and von Saalfeld and Dobson and Gladden.

Figure 4

A: Rest: Arterial inflow= venial outflow, veins are filled with blood and valves are open; B: Muscle contraction: neg. arterial inflow, veins are compressed, blood is expelled towards the heart; C: Relaxation; high arterial inflow, venous system gets filled with blood → when pressure and volume are normal --> venous outflow (increases until it reaches the same level as arterial inflow if there is enough time between contractions (see arrows))

This figure was obtained from: Tschakovsky, M. E., & Sheriff, D. D. (2004). Immediate exercise hyperemia: contributions of the muscle pump vs. rapid vasodilation. Journal of Applied Physiology (Bethesda, Md. : 1985), 97(2), 739–47. doi:10.1152/japplphysiol.00185.2004

1.1.2.6 Muscular plasticity and muscle protein turnover

Skeletal muscle is adapting with its metabolic, structural and contractile properties very sensitive to changing demands on its system. For example, by performing aerobic exercise (regularly running or swimming) the quantity of mitochondria in fibers will increase by de novo biogenesis to raise endurance of the muscle, nevertheless the muscle will not hypertrophy. Performing high resistance exercise, the cross sectional area of muscle fibers will increase but mitochondrial density may not increase (20). As mentioned above the skeletal muscle is adapting to change demand by turning over the proteins composing skeletal muscle fibers. As shown in figure five, every gene can run through modified expression via transcription and produce a pre-mRNA transcript. This primary RNA product gets modified into mature mRNA and is used as a blueprint for protein synthesis, which is called translation.

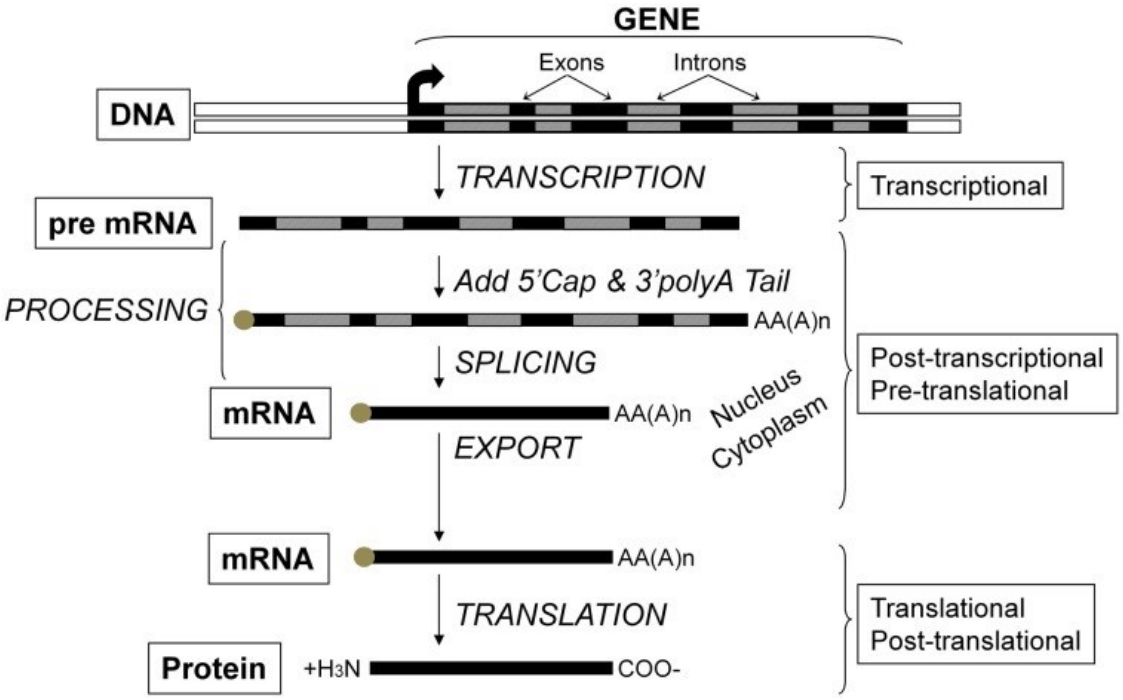

Figure 5 Main steps in gene expression regulation and genetic information chain. Protein synthesis and degradation determine the level of proteins expressed in the cell. Protein synthesis can be regulated by various processes in different levels: transcriptional, post-transcriptional, pre-translational, translational and post-translational.

This Figure was obtained from Baldwin, K. M., Haddad, F., Pandorf, C. E., Roy, R. R., & Edgerton, V. R. (2013). Alterations in muscle mass and contractile phenotype in response to unloading models: role of transcriptional/pretranslational mechanisms. Frontiers in Physiology, 4, 284. doi:10.3389/fphys.2013.00284

1.2 Exercise

Any intentional physical activity for preserving or improving physical fitness is called physical exercise. Studies have shown that constantly performed exercise prevents diseases of the affluence such as diabetes type 2, cardiovascular disease, obesity and heart disease (21,22). Health is influenced by every single fraction of physical fitness (i.e. muscular fitness, body composition and cardiorespiratory fitness). Humans having overall and/or abdominal obesity have an increased risk for lower health outcome than those with high free fat mass (they have a low risk for all-cause mortality) (23). Trained healthy people with a higher level of cardiorespiratory and muscular fitness have a lower risk for poorer health and even with a preexisting disease a lower risk for clinical events (23). Studies have shown that a weekly volume of 1000 kcal physical activity (a combination with varying intensity of physical activity and exercise (24)) reduces rates of cardio vascular disease and premature mortality (23).

1.2.1 Exercise: Types, duration, effects

There are three types of exercise rationed by their effect on the human body:

- Aerobic exercise: particularly for developing V•O2max (e.g. by swimming, running, hiking)
- Anaerobic (resistance) exercise: particularly for increasing muscular strength, endurance and physical function (e.g. weight training and sprinting)
- Flexibility exercise: stretching and extending muscle and improving range of motion of joints

By mixing the frequency, intensity and duration of chronic exercise the maximum training effect is reached. There are different kinds of exercises developing muscle strength and endurance: isometric (static) and isotonic or isokinetic (dynamic) exercise. For example more activity increases muscle strength and endurance (progressive overload principle) (26). Muscular strength improvement is best achieved by high resistance exercise. I.e. few repetitions conducted with maximum or near maximum force. The combination of more training repetitions with lighter weight leads to a gain in muscle endurance. The two training types improve to a certain point endurance and strength. This is always seen under the maxim, the lower the overload

stimulation the lower is the training effect and vice versa. It has also to be taken into consideration that every major muscle group of the body is trained, i.e. training the arms will have few or no effect on the legs and vice versa (25). To maintain physiological muscle strength and endurance exercise has to be performed on a regular basis. Just after 2 weeks of no training there is a significant decrease in cardiorespiratory fitness; in fact the pretraining fitness level is reached after 10 weeks to 8 month of no training (26).

1.2.2 Effects on the cardio-vascular system

The effects of regularly performed physical exercise on the cardio-vascular system are, improvement of the cardiovascular function as seen in enhanced maximum cardiac output and enhanced maximum oxygen consumption and increased blood flow capacity on cardiac muscle (27). Further exercise training can change the function and the vascular structure of vascular cells, e.g. exercise training generates shear stress (via increased blood flow) over the endothelium highly improving endothelium-dependent vasodilatation (27). Laminar shear stress induces releasing of vasoactive substances (e.g. nitrogen monoxide (NO) and prostacyclin (PGI_2)); the substances lower the permeability to plasma lipoproteins, leukocyte adhesion and prevent muscle cells from proliferating and migrating (28). In conclusion, high laminar shear stress blood flow is important for homeostasis in the vascular system by inducing signal transduction, gene and protein expression. Furthermore it has a key role in atherosclerosis prevention by regulation the vascular tone and keeping the endothelial cells in good function (28).

1.2.3 Effects on the musculoskeletal system

Skeletal muscle can adapt specifically to various stimuli by changing its mechanical and metabolic properties. Resistance exercises induces an increase in muscle size and strength and endurance exercise increases the oxidative capacity of the muscle without hypertrophy. In skeletal muscle autophagy (degradation of damaged cell parts/mitochondria) is activated via physical exercise (29,30). Autophagy is vital for muscle homeostasis and therefore exercise induced autophagy may contribute to beneficial effects of physical exercise (31).

Exercise as a countermeasure in spaceflight or to muscle atrophy

Bed rest studies have demonstrated that high-resistance exercise is a good countermeasure to disuse induced muscle atrophy and performance loss (32). Bamman et al showed in a 14 days bed rest study that by performing daily five sets x 6–10 repetitions to failure of constant-resistance concentric and eccentric plantar flexion is adequate to prevent muscle power loss (33). In a 30 day bed rest study (34) subjects were divided into three groups: no exercise, isotonic exercise and isokinetic exercise. It was shown that the isokinetic group (isokinetic knee flexion-extension) increased knee extension power by 27% and in contrast the no exercise group had a 16% decrease in power. The isotonic exercise group could keep their pre bed rest values. This highlights the importance of the specificity of training, since the isokinetic exercise group had the most benefit. Another 14 day bed rest study emphasizes this (35). The subjects daily performed five sets of leg press exercise to volitional fatigue (6-10 repetitions). An isokinetic concentric-eccentric knee-extension protocol was used to test the subjects before and after the bed rest. The non-exercise and exercise group showed no differences but both had decreased angle-specific torque (by 18%) and power fell (by 20%). Bamman et al. argue that on Earth locomotion needs constant external loaded contraction and therefore inflight exercise countermeasures should attach more importance to constant external loading (35). In conclusion: the perfect exercise program contain both isometric and isotonic exercise and high-intensity exercise is best for retaining limb muscle (32).

1.2.4 Whole body vibration training

Doing exercise on a vibrating platform is called whole body vibration (WBV) training. The platform generates vertical sinusoidal vibrations. The construction of the platform and the acceleration (which is determined by the frequency and the amplitude) define the characteristic loading of a vibration plate. There are two types of plates. First platforms turning around a fulcrum and thereby producing alternating forces on the right or left food and second, whole vertical vibrating platforms, generating simultaneous continuous forces on both feet. There are two different types of vibrating platforms, i.e. plates producing accelerations greater or lower acceleration than the gravity acceleration. The stimulation of the primary ending of the muscle spindles by the vibration plate leads to muscle contractions by activating the alpha-motor

neurons. The effectivity is yet unclear. Some WBV Studies have shown that WBV training is linked to an increased maximum strength and muscular power (36). Since muscle strength is directly linked to mobility and balance, WBV could have valuable effects in mobility and balance. Therefore WBV might be a good training for older people to improve their mobility and muscular strength (36).

But there are also findings suggesting that WBV training compared to the same exercises without WBV provides only little extra effects on muscular strength (the reason might be the quite low load imposed on the musculoskeletal system) (37). Another reason might be that the ideal training settings to induce max effects on the musculoskeletal system are yet unidentified (38).

Picture 1 A study probate is doing WBV training during the bedrest session.

This pictures was provided by ESA.

1.3 Immobilization

Skeletal muscle is the key tissue in many ways to life. It is highly adaptive to both in- and external influences. Microgravity (whether simulated or not) and disuse i.e. low mechanical load or inactivity, decrease muscle mass and strength. Increased mechanical load reversely induces muscle hypertrophy (39). Spaceflight with the lack of gravity is a parade example for mechanical unloading of muscle. On earth there are several models simulating spaceflight. Bed rest, especially 6°-head-tilt-down bed rest serves as the best model simulating the microgravity effects during spaceflight on cardiovascular and skeletal muscle and the reaction on changed circumstances (40). The musculoskeletal changes documented during bed rest, i.e. decreased BMD, increased bone resorption, increased Ca excretion, negative Ca balance and decreased muscle mass and strength have been found to be qualitatively similar to those occurring in spaceflight (41). In 6°-head-tilt-down bed rest muscle mass decreases very quickly. After a 7 days study with this model, subjects had lost 3% of their tight volume (42). The quadriceps and calf muscle mass decreased up to 30% after 90-120 days bed rest (43). In general, muscles of the lower extremity are more affected by disuse than the muscles of the upper limb and calf muscles are most prone to it (43).

1.3.1 Disuse and its effect on skeletal muscle

There is no doubt that skeletal muscle mass and fiber cross-sectional area is lost when muscles are exposed to disuse caused by either hindlimb suspension, joint immobilization, spinal cord injury, casting or spaceflight (44). During disuse, with intact nerves, in the initial one to two weeks skeletal muscle mass is lost rapidly; then the rate of lost decreases until a new equilibrium is achieved (44). This equilibrium is maintained until a stimulus, for example mechanical loading, stimulates muscle growth. Disuse effects on skeletal muscle includes a reduction up to 6-24% in muscle mass and strength (43) and various other changes linked to disuse (45). Nevertheless, the number of muscle fibers stay the same. In contrast, in age-related atrophy additionally the total number of muscle fibers decrease (46). Muscle atrophy is not just a simple loss of muscle mass and strength. It is an accurate controlled process which finally leads to reduced protein content, lower force production, a higher fatigability and lower insulin

sensitivity; in fiber types I and II a loss of capillary density and an expression of glycolytic phenotypes occurs (45).

In diseases such as cancer, AIDS, COPD cachexia (disease induced atrophy) is seen. In cachexia there is not only a disuse atrophy but also complex cytokine and inflammatory responses inducing signaling cascades and gene transcription. Injuries frequently come along with immobilization and are often accompanied by the side effect of muscle loss by mechanical unloading of muscle mass. This diploma thesis will focus on disuse atrophy.

Spaceflight

Microgravity removes most of the mechanical loading on muscles and is the main cause for muscle degradation in spaceflight (SF) (47). Data from previous SFs suggest that microgravity equilibrium of the limb muscle is reached after 180 days in space (32). Muscular atrophy equals lost power. Astronauts had a decline in muscle mass and maximal explosive power but their ability to generate power had a larger decline than the loss of force. For instance, after a 31 days of SF lower limb extensor maximal explosive power declined by 54% while force declined by 11 % (48).

Morphological changes induced by spaceflight: in general slow fibers are more sensitive to SF than fast fibers (49). Additionally extensors are more sensitive to SF than flexors and therefore losing more muscular mass (e.g. triceps surae compared to musculus tibialis anterior) (32). In a study soleus muscle fibers of rats after 14 days of SF were compared with a ground-based control group. The myonuclear number per millimeter declined significantly in type I fibers but no significant change was observed in type I+II and II fibers (49). The cross-sectional area (CSA) of fibers decreased significantly in type I and I+II fibers, whereas the CSA of type II showed no significant change (49). The total amount of type I fibers were reduced and type IIa and IIx fiber numbers increased (49). These changes increase fiber velocity which counteract a part of the loss in maximal explosive power (32)

1.4 Molecular mechanisms of muscle hypertrophy and atrophy

Skeletal muscle adopts elegantly to changing demands. When being overloaded mechanically (e.g. strength training) or stimulated by testosterone or β-adrenergic agonists (anabolic hormones), skeletal muscle hypertrophies. Muscle atrophy has multiple reasons, to name a few: aging, bed rest, spaceflight, diseases and hormonal stimulation by corticosteroids. As a reason for changes in protein turnover, cellular and nuclear turnover have to be considered as well. Muscle protein synthesis is controlled by two major signaling and several other pathways. The IGF-1-PI3K-AKT-mTOR pathway is a positive regulator and the myostatin–Smad2/3 pathway is a negative regulator of muscle protein synthesis. Protein degradation has two main signaling pathways: the proteasomal and the autophagic–lysosomal pathway. Various atrophy associated genes or atrogenes are involved in these pathway. FOXO3 and NF-κB are controlling them (27). In conclusion, the homeostasis of skeletal fiber muscle protein is given by the relation of protein translation activity to protein degradation activity. In hypertrophy, there is relatively more protein translation, in atrophy there is relatively more protein degradation (20).

1.4.1 Signaling pathways controlling muscle growth

1.4.1.1 IGF-1-PI3K-AKT-mTOR

The IGF-1-PI3K-AKT-mTOR pathway is an important regulator of muscle hypertrophy because of the downregulation of protein breakdown and the support of muscle growth (50). When activated, protein synthesis is increased by up-regulating translation initiation factors: Phosphatidylinositol 3 kinase (PI3K) (when activated by IGF1) phosphorylates in the membrane phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphatea and thereby builds a binding site for AKT. Activated AKT phosphorylates the mammalian target of rapamycin (mTOR) kinase, thereby activating it. Activated mTOR activates p70S6kinase and eukaryotic translation initiation factor 4E binding protein 1 vie phosphorylation. Both of them affect the translation. AKT activation seems to play an important role in cellular signaling pathways regulating atrophy and hypertrophy (45). More precisely, AKT modulates transcription factors the ubiquitin–proteasome system and the autophagy–lysosome pathway by phosphorylating Forkhead box (FoxO). Relevant for skeletal muscle are FoxO1, FoxO3 and FoxO4. When FoxOs are phosphorylated, they get transferred faster from the nucleus into the

cytoplasm. When AKT is down regulated, the number of phosphorylated FoxO located in the cytoplasm falls and FoxOs in the nucleus rise (51). In times of disuse AKT is not activated and so FoxO induces atrogin-1 and muscle-specific RING Finger protein1 (MuRF1) expression causing muscle atrophy. Over-expression of FoxO3 in human skeletal muscle causes fiber atrophy and decreases muscular mass (52). Muscle loss is prevented by up regulation of atrogin-1 expression during atrophy (52). FoxO transcription factors regulate the activity of muscle atrophy F-box (MAFbx/atrogin-1) and MuRF1 (53). FoxO acts on cell metabolism, apoptosis and the cell cycle (54).

Changes in the IGF-1-PI3K-AKT-mTOR pathway correlate with changes in the ubiquitin proteasome ligases, MAFbx and MuRF1 (55). By activating the IGF1-pathway FoxO translocation is highly down regulated. Reversely, if the IGF1-pathway is down regulated, FoxO translocates into the nucleus, inducing proteolysis and inhibiting muscle growth (53). Dirks et al. showed that transcript levels of MAFbx and MuRF1 in humans increased after being immobilized for 5 days without any intervention (56). mTOR has been identified as one of the key regulators of processes in the cell, but more research has to be done to understand the whole interactions of mTOR (45).

1.4.1.2 Myostatin

Myostatin (growth-differentiation factor 8) is part of the transforming growth factor β family and is manly expressed in muscular tissue (57). It has been shown that myostatin downregulates muscle mass, e.g. myostatin knockout mice had over 200% more muscle mass than wild-type littermates (57). Another example is the "double muscled" cattle, i.e. mammals without myostatin gene, either by natural mutations or knockout (58). Inhibition of myostatin gene expression, skeletal muscle tissue hypertrophied by 13-30% (59). To find exact correlations between myostatin and atrophy is challenging. Myostatin seems to be an inhibitor of the AKT-mTOR pathway and an up regulator of the ubiquitin proteasomal pathway via FoxO (60). Myostatin inhibits the activity of MyoD and initiates myoblast to enter the G0 phase rather than inducing cell apoptosis or differentiation (61). Mice having been in space for 11 days and humans after chronic disuse show elevated levels of myostatin mRNA (49,62). Subjects being 25 days in a head down bed rest study showed elevated serum myostatin levels by 12%(63).

Another study showed after 5 days of immobilization a rise in myostatin transcript levels, but the same probates had dropped myostatin levels after 14 days (64). Myostatin given intravenously to mice causes muscle atrophy (65). Follistatin, an inhibiting extracellular binding protein, binds to other members of the TGFβ family (for example to activin A, an opponent to muscle growth) and thereby might induce hypertrophy. Myostatin and activin A interact together and so activate a receptor complex which contains two receptors, activin receptor 2 (ACVR2 and ACVR2B), and activin receptor-like kinase 2 and 5 (ALK4 and ALK5) (50). The soluble version of ACVR2B acts as a myostatin and activin A inhibitor causing in mice muscle hypertrophy (50). The signaling pathway of Myostatin/activin A is: they phosphorylate and induce a nuclear translocation of Smad2 or Smad3 transcription factors and build heterodimers with Smad4 (50). The way Smad2/Smad4 and Smad3/Smad4 complexes work are not yet understood but it might be that the Akt–mTOR pathway is influenced by myostatin/activin A signaling (66).

1.4.2 Signaling pathways in muscle atrophy

After the protein is selected for degradation several pathways exist .Most of the degradation is done by the ATP-dependent ubiquitin-proteasome pathway (see Figure 7), but there are more intracellular proteolytic paths like the lysosomal pathway and calcium activated proteases.

1.4.2.1 Ubiquitin proteasome pathway

The ubiquitin-proteasome pathway is the main degradation way for myofibril proteins in different forms of muscle atrophy (67). The degradation of the myofibril proteins occurs in three steps. Firstly is the proteolysis process which is regulated by calcium activated proteolytic enzymes (e.g. calpains and caspases) (68). Secondly is the ubiquitin-conjugation process (mainly a N-end rule patway with a three step enzyme reaction wich binds the protein with poly-ubiquintin molecules (Figure 7)). Thirdly, the ubiqutinated protein is transferred in the 26S proteasome complex where it is degradet into free amino acids. Figure 7 shows how depending these steps are on ATP.

MuRF1 and MAFbx/atrogin-1 are two crucial E3 ligases being expressed specifically in cardiac and skeletal muscle and acting upon it (69). MAFbx and MuRF1 are overexpressed in skeletal

muscle in various disuse models (69) and might play a role in initiating of atrophy (70). For example MuRF1 knockout mice had, 14 days after denervation of the tibialis anterior and gastrocnemius muscle, 36% sparing of muscle compared to 14 days denervated wild-type mice (69).

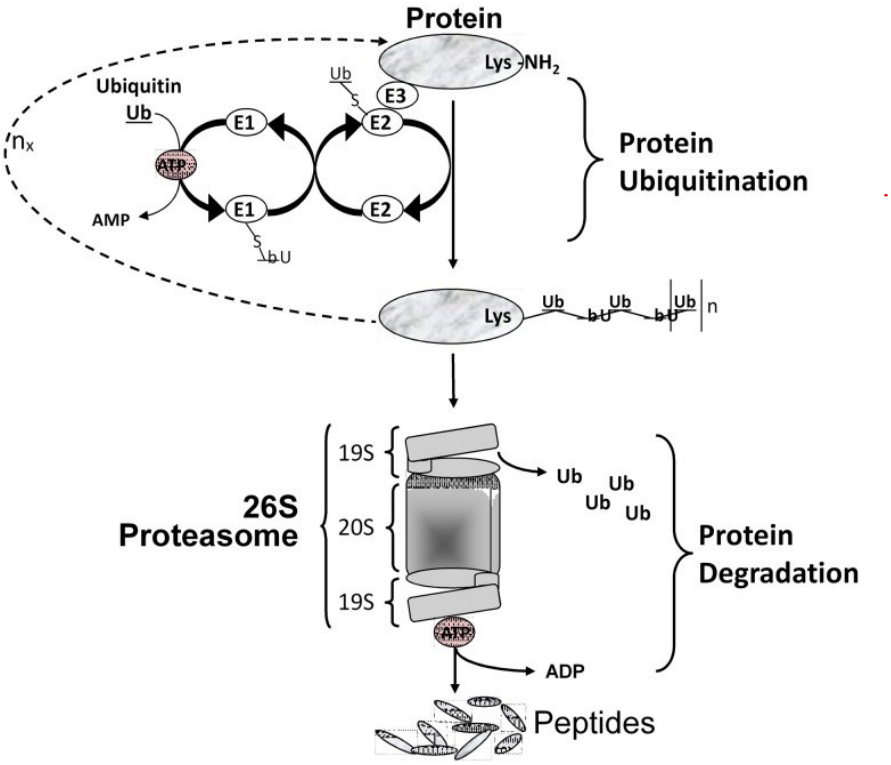

Figure 6 Degradation of proteins by the ubiquitin/proteasome pathway. E1 (Ub activating enzyme) activates Ub E2 (Ub carrier protein) takes the activated Ub and binds it with assistance of E3 (Ub-protein ligase) to an amino group of the protein. More Ub molecules get bound to the subjoined Ub (link to a lysine NH2 group) building a polyubiquinated protein. This protein can be detected by the 26S proteasome system and gets degraded.

This Figure was obtained from: Baldwin, K. M., Haddad, F., Pandorf, C. E., Roy, R. R., & Edgerton, V. R. (2013). Alterations in muscle mass and contractile phenotype in response to unloading models: role of transcriptional/pretranslational mechanisms. Frontiers in Physiology, 4, 284. doi:10.3389/fphys.2013.00284

1.4.2.2 The autophagy-lysosome system

Autophagy is an important player in the turnover of cell components whether the cell is under normal circumstances or is exposed to different stimuli (e.g. cellular stress and cytokines) (71). With macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy, three mechanisms have been found who are in charge for the autophagy-lysosome system in mammals (Figure 8). There are some findings suggesting that microautophagy happens in skeletal muscle (72) but more research has here to be done. The same counts for CMA, its part on muscle homeostasis or atrophy is yet unclear, but there are findings highlighting its potential role in different diseases (31). During fasting skeletal muscle has the most vesicle formation compared to other tissues. In type I muscle fibers the concentration of autophagosomes is higher than in type II muscle fibers (71). Active FoxO3 overexpression induces muscle atrophy by autophagy (73). It seems that autophagy is not just a non-specific depletion pathway, but is also able to be selective. For example mitophagy: the specific autophagy of mitochondria (31) e.g. in muscle atrophy (74).

In conclusion we can say that homeostasis of the autophagy-lysosomal pathway is essential for maintaining skeletal muscle; an imbalance in this system is a cardinal cause in many muscle diseases (31). Overactive autophagy causes degradation in muscle cells resulting in muscle atrophy, vice versa insufficient autophagy causes muscle weakness by being unable to remove damaged or abnormal cell components (31). There are also findings suggesting that a minor but chronic imbalance in the autophagic system has a large share on causing sarcopenia (75).

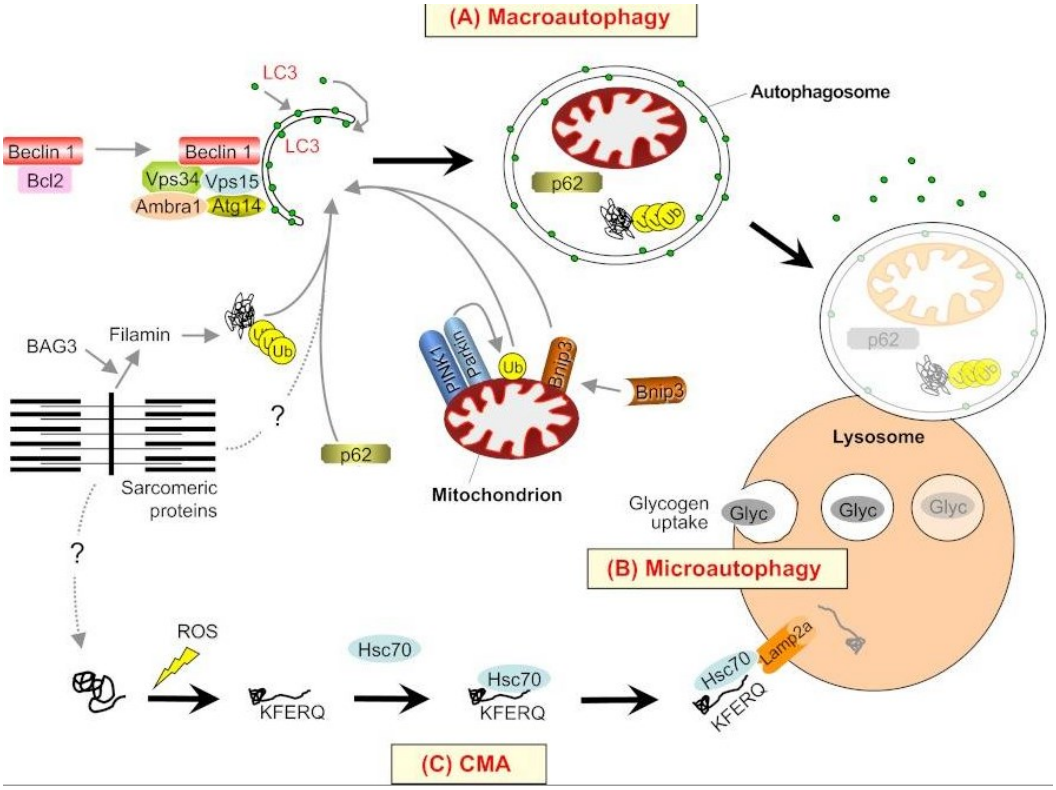

Figure 7 Involvement of macroautophagy, microautophagy and chaperone-mediated autophagy on skeletal muscle maintenance. (A) Macroautophagy is activated by activation of a complex (including Vps34, Beclin 1, Vps15, Ambra1 and Atg14) triggering LC3 to bind to the isolation membrane of the emerging autophagosome. Mitophagy needs PINK1-parkin complex and Bnip3 factors. To be able to degrade proteins, they are polyubiquinated and p62 scaffold protein transports them to the autophagosome. (B) Microautophagy comprises directly degradation of small amounts cytoplasm and Glycogen in lysosomes. (C) CMA: Damaged proteins (e.g. by reactive oxygen species) express the specific KFERQ motif (an amino acid sequence). Hsc70 chaperone, can now identify them and via Lamp2a receptor the proteins are incorporated into the lysosome. The dotted lines represent yet unknown molecular mechanism pathways in skeletal muscle

This Figure was obtained from: Bonaldo, P., & Sandri, M. (2013). Cellular and molecular mechanisms of muscle atrophy. Disease Models & Mechanisms, 6(1), 25–39. doi:10.1242/dmm.010389

1.4.2.3 Inflammatory cytokines and NF-κB signaling

Inflammatory cytokines with TNFα leading the way, activate NF-κB transcription factors in the skeletal muscle (76). Normally IκB is bound to NF-κB and inactivating it. In states of inflammation with raised TNFα, TNFα activates the IκB kinase (IKKβ) complex which finally leads to degradation of IκB. NF-κB now can translocate into the nucleus and activates transcription of NF-κB mediated genes (76). The NF-κB signaling pathway boosts the proliferation and fusion of myoblast and inhibits myogenesis (77). When TNFα and other inflammatory cytokines level raise, they lead to insulin resistance and an inhibition of the IGF1–Akt pathway (78). Several studies have been underlining the communication between the IKKβ–NF-κB and Akt–FoxO pathway, but more research has to been done to understand their influence on muscle atrophy. Another member of the TNF superfamily causing muscle atrophy is TNF-like weak inducer of apoptosis (TWEAK) (79). TWEAK inhibits PI3K/Akt signaling pathway and activates ubiquitin-proteasome and NF-κB systems (79).

1.5 OMICS analysis

The suffix –omics is a neologism referring to a wide field of studies in biology. The aim of OMICS is to analyse large sets of molecules instead of just studying isolated biological molecules (80). OMICS are used: to explore the connection between environmental stress and human adaption/response; to investigate the underlying complex molecular organization; to scrutinize biological pathway dynamics at work; to detect biomarkers and thereby to increase the sensitivity of OMICS and to develop new countermeasures. Although OMICS technologies are developing rapidly at the present time, five OMICS technologies are the most developed: genotyping, transcriptomics, epigenomics, proteomics and metabolomics (80).

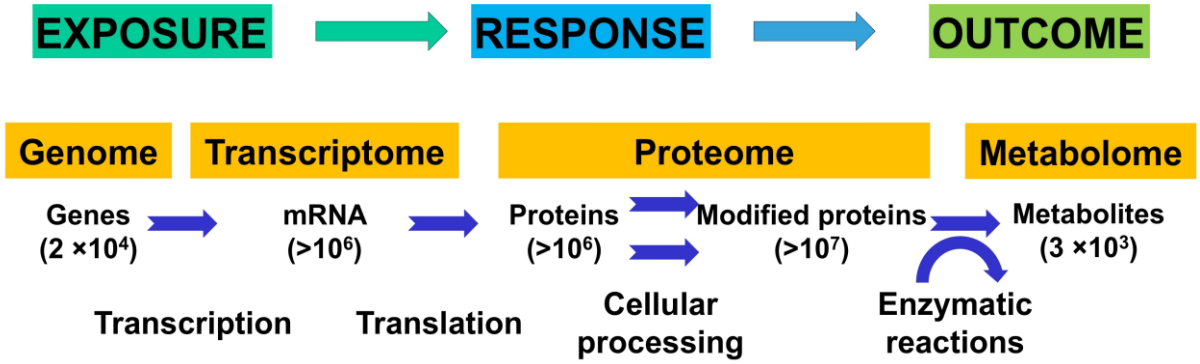

Figure 8 shows the connection of the several OMICS technologies, how they are related and their biological basis.

1.5.1 Genomics

1.5.1.1 Genotyping

Genotyping aims to identify the physiological function of genes and their specific role in disease vulnerability (81). The knowledge gained by the human genome project (HPG) and technological development led to the invention of assays who could, in a single experiment, asses the variability in the DNA sequence of thousands of genes. Among different types (insertion/deletion of nucleotide base pairs and DNA copy number variations (CNVs)) of genetic variations, single nucleotide polymorphisms (SNPs) are the most explored with over 9 million identified SNPs (80). Since SNPs exhibit low mutation rate and are numerous in the human genome, they are used as a genetic variation marker in disease-gene associated studies

(82). Due to the low mutation rate and the haplotype structure and the linkage to imbalance in small genome regions tag SNPs (subset of informative SNPs) can be used as genotyped proxies for identifying disease associations and human traits (83). Still studies have to be done to examine if associate SNPS influence the structure or function of DNA, RNA, proteins and influence phenotypes. CNVs are defined as "segments of DNA that are 1 kb or larger and present at variable copy number in comparison with a reference genome" (84). Deletions, duplications and deletions are structural genomic variations causing CNVs. CNVs located in gene promoter regions can affect gene expression. When located proximal to genes, CNVs might disturb the gene expression and the "histone code; exon located CNVs might lead to mis-spliced mRNA with damaging effects on protein expression" (84).

With genome-wide association studies (GWAS) it is now possible to cover in a single assay the entire genome and to discover unknown chronic disease associated polymorphic variants (80).

1.5.1.2 Transcriptomics

The quantity of specific mRNA transcripts is linked to expression levels of the corresponding genes (85). In gene expression profiling the level of gene expression is characterized, since specific forms of mRNA and their expression levels are parameters providing gene expression information (86). Gene expression profiling is used to identify and characterize the compound of mRNA being existing in a particular sample. Gene expression profiling is used to attribute varieties in mRNA mixtures from different groups of individuals to the groups phenotypic differences (87). Compared to the genome, the transcriptome is very changeable in the course of time, among different cell typed and alters with responses to changes in the environment (80). By giving a quantitative overview of the mRNA transcripts in a sample, gene expression profiling can be utilized to define the response in gene expression to changed environmental conditions.

1.5.1.3 Epigenomics

In epigenomics epigenetic processes are investigated on a genome wide scale (88). Despite the number of epigenetic mechanism detected is continuously growing (e.g. gene expression interference by microRNA and siRNA), epigenomics is primary based on two mechanisms: DNA methylation and histone modification (it is believed that all the named processes interact together) (80). When a methyl group is attached to cytosine in a CpG dinucleotide, the process is called DNA methylation (it is differentiated if methylation is global or CpG island specific) (80). In the human genome 70% of the CpG dinucleotides are methylated, yet hypermethylated gene promoter region located CpG island are connected to gene silencing (e.g. x-chromosome inactivation and tissue specific gene expression) (89). Whereas hypomethylation isn't still well understood (there might be a link to chromosomal instability) (90). The structural packing of DNA in the chromatin complex includes histone proteins. By being altered post translational e.g. by acetylation and methylation, the altered histones might regulate chromatin structure and as a consequence gene expression (91).

1.5.1.4 Proteomics

The function of proteins in a biological system is explored with proteomics. The challenge is: nearly everything in the human body is composed of /by proteins and the high variability of these proteins (92). Proteins located in the intra- and intercellular space and their quantity determine the function of a cell. Despite every protein is made out of mRNA precursors, it is impossible to forecast the quantity of specific proteins just by analyzing the gene expression alone. All proteins in a specific cell or tissue are the proteome. The proteome adapts to changes in its environment and is different between cell types. Therefore technologies are needed that have the ability to detect a broad range of proteins in samples of various origins (93). The existing proteomic technologies can be divided into two approaches: mass spectrometry and proteins microarrays. One of the main goals of proteomics is to identify proteins including post translational modificated proteins and the interaction of proteins in protein-complexes (80). The quantification of the protein abundance is another goal of proteomics. Protein translation and degradation determines protein expression levels in cells, for that reason the abundance of a specific protein might be linked to its part in cell function.

1.5.1.5 Metabolomics

Metabolomics is the study (of samples like plasma, urine or saliva) of the current metabolic status given in a biological system (94). Products of interactions of various factors such as genetic, environmental, lifestyle and gut microbial are called the metabolic phenotypes (95). Metabolites (e.g. vitamins or lipids) build up the metabolome; they play a role in cell metabolism by being connected to different biological molecules via determined metabolic pathways (94). The metabolome is composed of a large number of chemical structures and changes over time (80).

1.5.1.6 Bloodomics

For certain examinations it is inevitable to take biopsies, but due to the risk of unwanted harms or hard access to the tissue (e.g. brain and heart), they play a minor role in daily clinical routine. Searching for new diagnostical approaches, blood has moved into the focus. Taking blood is an everyday clinical routine afflicted with a low risk of harms. Blood has contact with almost every tissue in the human body and about 80 % of the transcriptome of examined tissues can be found in peripheral blood cells (96). Therefore blood might be used as a surrogate biopsy material. In bloodomics, the OMICS technology is used to gain information about distinct parts and organs of the body. Since blood reflects the physio/pathological status of tissues, a change in a specific organ leads to an equal change in the blood components. For example: tissues and dying cells release intracellular proteins, which get into the blood; with proteomics/metabolomics they can be detected. When the tissue or the cells release mRNA or DNA fragments they can be measured with Transcriptomics (97). Nowadays we assume that regulatory systems for specific cellular transcriptomes affect the targeted cells but also have systemic effects on other cellular types. It seems that many regulatory systems are linked, even if they do not interact directly, so a change in one system can be detected in the other.

2 Aims and Objectives

The aim of this 21 days bed rest study is to study how the muscular system of the human body is affected by bed rest and if any differences can be observed depending on the type of intervention (only bed rest and bed rest and exercise). The coherence between immobilization and the loss of skeletal muscle is known for a long time but yet not fully understood. With the data from this bed rest study we want to understand the relationship between environmental stress (in this case immobilization) and human response (in this study muscle loss), adaption and deconditioning. More precisely we try to understand the underlying complex molecular organization and want to elucidate the dynamic of biological pathways at work. Additionally we try to find new genes involved in the regulation of muscle homeostasis and evaluate countermeasures. We hope that the results are useable for medical fields such as cardiology, geriatrics and immobilization.

The main objective of this study is to examine the microscopic molecular changes in cells that lead to macroscopic changes in the volunteer's body. This was observed by collecting blood samples and measuring the changes in the cells gene expression (changes in expression of cRNA). According to the hypothesis that all physical processes arise from genetic control (transcription and translation, changes in gene expression lead to different gene products and thereby lead to macroscopic changes). Consequently changes in the environment should lead to adaption of intracellular gene expression and therefore lead to changes in cellular gene products (e.g. enzymes). As a result cells alternate (cellular hyper/hypotrophy, mitosis, apoptosis). The entire boy might change/adapt to the new conditions: if the intracellular substance reaches same/different cellular compartments (e.g. substances attain into the blood vessels and are transported throughout the body) they can induce there changes (e.g. if the substance is a peptide hormone).

By measuring the transcriptome (all detectable transcripted genes (mRNA) given at a present time (before, during and after changes in the environment)) it should be possible to understand the adaption process occurring in the body. If we fully understand the role of a protein in various pathways, and an increase of mRNA of this protein is measured we might predict its up-regulation. This diploma thesis focuses on muscle loss by measuring RNA levels before, during

and after bedrest comparing two study groups (with and without exercise). The goal is to understand the underlying pathway of muscle hyper/atrophy.

3 Methodology

3.1 Subject's recruiting and medical selection

12 health male volunteers participated in this 21-day 6-degree head down bed rest study (to simulate spaceflight induced fluid shifts, see section 1.2.2), which was sponsored by ESA. They were recruited following public advertisements. Strict inclusion and exclusion criteria were used to select these subjects.

The study was carried out at MEDES (located in Toulouse, France). MEDES Space Clinic and specialized departments of the Rangueil and Larrey Hospitals (Toulouse) carried out the test on the candidates. The ethic boards of MEDES approved the study which is conform to the 1989 WMH declaration of Helsinki.

3.1.1.1 Subject suitability:

Volunteers had to fulfil following conditions to be able to participate in the experiment:

They had to read carefully an information document, this was verified. Afterwards the volunteers hat time to ask any question they had regarding the experiment. All the answers were replied until complete satisfaction was reached. Then they hat to sign the specific Information and Consent Form.

The test results were checked and the volunteers were controlled if they fulfil all inclusion criteria and no non- inclusion criteria.

3.1.2 Inclusion criteria

- Healthy male volunteer (see below the description of medical tests and laboratory analysis performed at the selection visit)
- Age 20 to 45
- BMI (weight Kg/ height m^2) between 20 and 26
- Height between 158 and 190 cm
- No personal nor family past record of chronic or acute disease or psychological disturbances which could affect the physiological data and/or create a risk for the

subject during the experiment,

- Fitness level assessment:
    - if age < 35 years: 35 ml/min./kg < VO2max < 60ml/min./kg,
    - if age > 35 years: 30 ml/min./kg < VO2max < 60ml/min./kg
- Active and free from any orthopedic, musculoskeletal and cardiovascular disorders,
- Nonsmokers, no alcohol, no drug dependence and no medical treatment,
- Covered by a Social Security system,
- Have signed the information consent,
- Free of any engagement during the three hospitalization planned periods.

3.1.3 Non-inclusion criteria

- Past record of orthostatic intolerance
- Cardiac rhythm disorders
- Chronic back pains
- History of hiatus hernia or gastro-esophageal reflux,
- History of thyroid dysfunction, renal stones, diabetes, migraines
- Past records of thrombophlebitis, family history of thrombosis or positive response in thrombosis screening procedure
- A positive result for phlebitis markers or the presence of one of these mutations
- Abnormal result for lower limbs echo-doppler
- History or active claustrophobia
- History of genetic muscle and bone diseases of any kind
- Bone mineral density: T-score ≤ -1.5
- Osteosynthesis material, presence of metallic implants
- History of knee problems or joint surgery/broken leg
- Poor tolerance to blood sampling,
- Having given blood (more than 8ml/kg) in a period of 8 weeks or less before the start of the experiment,
- Special food diet, vegetarian or vegan
- History of intolerance to lactose or food allergy (milk proteins...)

- Positive reaction to any of the following tests: HVA IgM (hepatitis A), HBs antigen (hepatitis B), anti-HVC antibodies (hepatitis C), anti-HIV_{1+2} antibodies
- Echocardiography: inappropriate thoracic acoustic window
- Subject already participating or in the exclusion period of a clinical research
- Refusal to give permission to contact his general practitioner
- Incarcerated persons
- Subject who, in the judgment of the investigator, is likely to be non-compliant during the study, or unable to cooperate because of a language problem or poor mental development
- Subject who has received more than 4500 Euros within 12 months for being a research subject
- Subject under guardianship or trusteeship

3.1.4 Medical tests description

The medical check-up was carried out at several clinic and involved:

3.1.4.1 At the Space Clinic:

- A medical and surgical history including habits life, alcohol, caffeine, tobacco consumption, previous medication,
- A complete clinical examination,
- A measurement of systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) in both supine position (after the subject has rested comfortably for at least 10 minutes) and standing position after 3 and 10 minutes (Stand Test). These tests will check the absence of orthostatic hypotension,
- A familiarization test with the Tilt table,
- A 12 leads electrocardiogram,
- A measurement of maximal oxygen consumption (VO_2 max test),
- An interview with a psychologist,
- A DEXA measurement of the bone density,

- An alcohol breath test,
- A biological screening:

3.1.4.2 **At Laboratoire Biopole**

(France – accreditation number 31.3.31158.1)

Biochemistry

Fasting glucose, sodium, potassium, chloride, calcium, HCO_3^-, total protein, albumin, total cholesterol, HDL cholesterol, LDL cholesterol, triglyzerides, urea, creatinine, total bilirubin, asparatate aminotransferase (ASAT / SGOT), alanine aminotransferase (ALAT / SGPT), alkaline phosphatase, gamma glutamyl transferase (GGT), uric acid, C-reactive protein (CRP), TSH.

Hematology

Cell blood count (CBC), platelets, reticulocytes, fibronogen, PTT, PT, phlebitis markers (anti thrombin III, S-protein, C-protein), molecular screening (research for the factor V Leiden mutation and the mutation 20210 of the prothrombin gene). If a subject fulfils a non-inclusion criteria a haematologist will give this information to him during a consultation.

Vitamins and mineral status

Fat-soluble vitamin: retinol, retinyl-palmitate, beta-carotene, apha-carotene, serum phylloquinone, alpha-tocopherol, gamma-tocopherol. Water-soluble vitamin status: erythrocyte glutatione reductase, vitamin B6, red cell folate, vitamin C, vitamin B12, 25 OHD (Vit D). Serum iron, ferritin, transferin saturation, transferin receptor, selenium, copper and zinc.
If a volunteers has at the time of the medical screening a deficiency in (25-OHD) Vitamin D and at least two months are left before the study starts, he will be treated with 1000 IU Vitamin D_3. If the subject doesn't fulfil this criteria, he will be excluded. All the subject will be supplemented during the whole study with 1000IU to avert seasonal variations.

Serology

Search for the hepatitis markers (A, B, C) and serological HIV. The subjects will be informed of the results before the test starts.

Urine drug screen

Nicotine, barbiturates, benzodiazepines, opiates and cannabis.

3.1.4.3 At Medes

Urinalysis

Urinalysis is made on strips: haematuria, leucocyturia, glucose, ketonuria, proteinuria, bilirubine, urobilinogen, nitrites, pH, and density.

3.1.4.4 At specialized Departments (Rangueil and Larrey Hospitals, Toulouse)

Venous deficiencies were excluded by echo-doppler measurements of the lower limbs in order to eliminate the venous deficiencies, chest radiography front and side, cardiac US scan tests were performed in order to make sure of the quality of the collected images (appropriate acoustic window).

3.1.4.5 Psychogical screening

The screening included a paper pencil test on biography and personal history of the subjects and a standardized test on personality. After the tests the subject were interviewed face to face. Tests and interview were carried out by an experienced psychologist.

The examinations have two purposes:

- To select out persons having psychopathological tendencies
- To select in volunteers having the best fitting profile for the experiment

3.2 Study design

This study was designed as a pilot study with an cross-over design .12 health male volunteers participated in this three sessions 21-day 6-degree head down bed rest study simulating space flight induced fluid shifts. Proteins help gaining muscle mass faster and exercise preserve muscular strength. To explore differences, the volunteers where divided into three groups (four

persons in each group). The first group only lay in bed, the second group performed WBV training, the third group had additionally to the exercise training daily doses of proteins. Apart from that the different groups received the same treatments and test.

In-between the 21-day bed rest sessions the volunteers had a four month recovery phase to get back their normal physical condition. After the recovery phase all group members entirely switched to another group. This was done twice (in total three sessions) to achieve the randomized cross over study design. To minimise falsifications the volunteers were allowed to perform just minor sports the last 10 days before the start of the study session.

3.3 Microarray analysis protocol

3.3.1 Step 1

A 3-mL non-fasting blood sample was collected by phlebotomy between 8 am and 12 pm (5 days before the start of the study, during the study on the days 2, 7, 14, 21 and on the first and second day after the study blood was collected). The collected blood was mixed in the tube and was stored at -20 °C within 1 h after collection.

Tools used: Tempus Blood RNA tubes (Applied Biosystems, Halle, Belgium).

In this diploma thesis due to financial issues the transcriptome data from two samples (prior bedrest and directly post bedrest) collected from 9 volunteers (3 per group) were taken.

3.3.2 Step 2

Total RNA was extracted ([1]) then RNA yields were reviewed ([2]). Globin mRNA was depleted from total RNA preparations ([3]).The integrity of the remaining globin-depleted RNA was determined ([4]).Samples were stored at -80 °C.

Tools used: ([1])Tempus Spin RNA Isolation kit (Applied Biosystems) according to the manufacturer's instructions; ([2]) NanoDrop Spectrophotometer (Isogen Life Science, PW De Meern, the Netherlands); ([3]) Ambion Globinclear kit (Applied Biosystems), carried out with the providet instructions; ([4]) Agilent 2100 Bioanalyzer using RNA 6000 Chips (Agilent Technologies, Diegem, Belgium).

3.3.3 Step 3

Complementary RNA was generated by amplifying and labeling the globin-depleted RNA ([1]). A RNA subsample (100-200ng) was reverse transcribed into complementary DNA ([2]). cDNA was transcribed into cRNA, at the same time cyanine 3-CTP was integrated. The singe-stranded, labeled cRNA was purified ([3]) and yield and specific activity were determined ([4])

Tools used: ([1]) Low Input Quick Amp Labeling (one colour) kit from Agilent Technologies; ([2]) T7-promotor primer and MMLV reverse transcriptase; ([3]) Qiagen's RNeasy mini spin columns (Qiagen, KJ Venlo, Netherlands); ([4]) NanoDrop spectrophotometer

3.3.4 Step 4

Aliquots of 1.65 µg cRNA were hybridized ([1]). The arrays were scanned ([2]) and then converted into text files ([3]). The gprocessed signal per probe was used for analysis. The latter signals were quantile normalized and $\log_2$-transformed ([4]). Replicated probes on the array were reported as median signals. After removing the control probes, 41000 unique probes remained. They were filtered to the intenseness of gene expression and mapped to an approved HUGO gene symbol.

Devices used: ([1]) 4x44K Agilent Whole Human Genome microarray slides (design 014850) for 17 h using the automated HS4800TM pro hybridization station (Tecan, Männedorf, Switzerland) according to the manufacturer's instructions; ([2]) Agilent DNA microarray scanner (G2565BA) and processed using Agilent Feature Extraction Software (Version 10.7); ([3]) by the Agilent protocol GE1-10.7-SEP09; ([4]) using GeneSpring 12.1 (Agilent Technologies)

3.3.5 Step5

Statistical analysis

Paired student t-test per probe was used. The Benjamini-Hochberg false discovery rate (BH-FDR) was used to adjust all p-values for multiplicity. If the false discovery rate p-value was below 0.05 and the absolute fold change was larger than two, a probe was called significant.

3.3.6 Step 6

Biological interpretation

All these steps generated data plots of expression values of 41000 probes. In Belgium the data got assessed there using Ingenuity Pathway Analysis (IPA version 8.0, Ingenuity® Systems, http://www.ingenuity.com) and the ontology term “muscular process” in the muscular system and function tree. 220 probes with a relation to skeletal muscle were detected. Each genes fold change was calculated with fold change =1, fold change <1 means up-regulation and fold change > 1 means down-regulation.

4 Results

Twelve healthy male volunteers, who fulfilled the criteria numerated in the methodology part, participated in this 21-day 6-degree head down bed rest pilot study. Their average weight was 69.8 ± 8kg, the average age was 34 ± 8 years and they had an average height of 1.76 ± 6 cm (mean ± standard deviation). Figure 9 illustrates the transcriptomic data gained from the whole blood samples, taken prior and post bedrest, of nine volunteers.

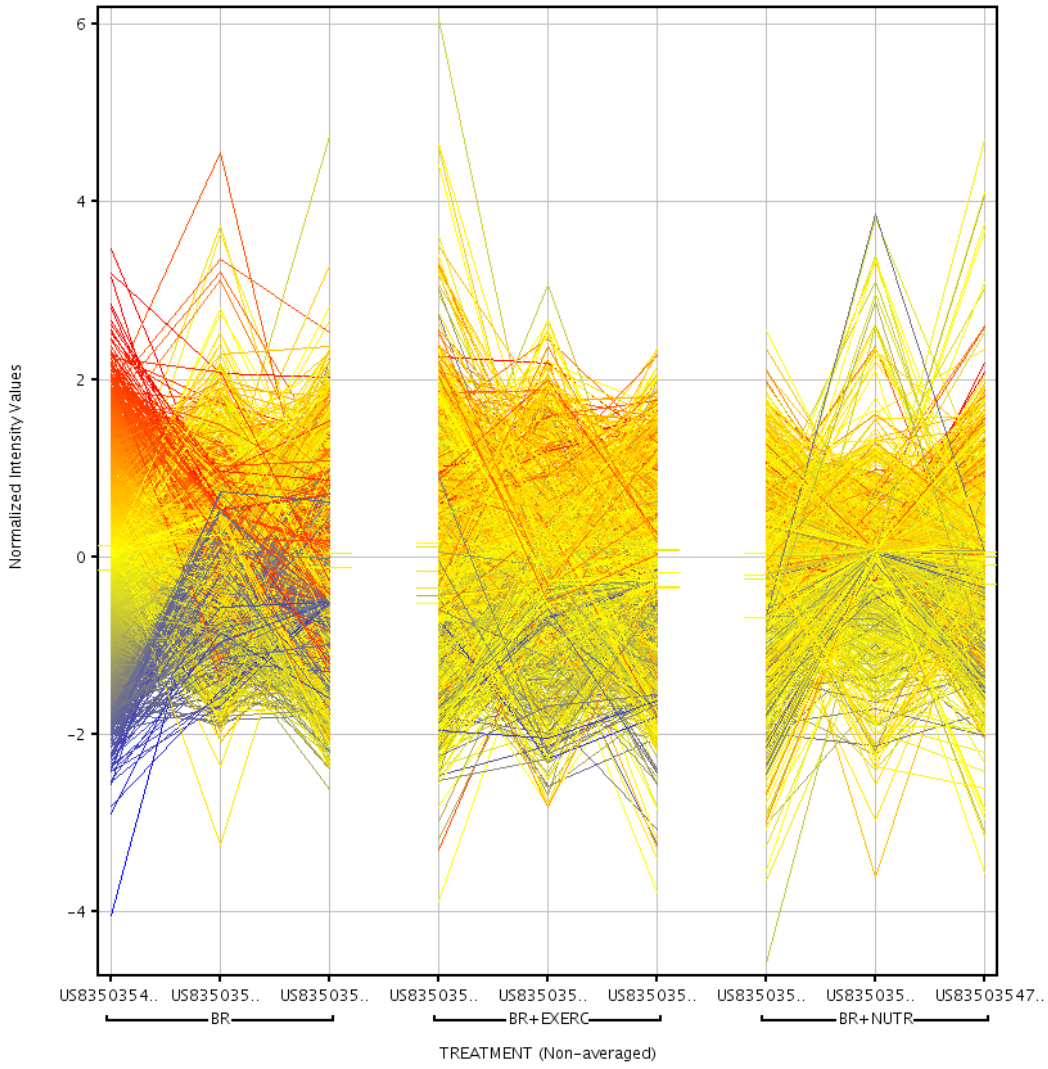

Figure 9: **Gene expression reaction.** Unpicked data of all recovered genes. Volunteers are coded by US-numbers. BR = bed rest, BR+EXERC = bedrest and exercise, BR+NUTR = bedrest, exercise and nutrition. Normalized intensity values are positive for upregulation and

negative for downregulation of the particular gene expression after treatment. Intensities of the same genes of different volunteers are linked by lines. Flat lines depict the same change in gene expression in different participants.

Using Ingenuity Pathway Analysis 220 genes were found coding for muscle proteins. Figure 10 shows the fold change values of the two study groups bedrest (colored blue) and bedrest and exercise (colored orange). The cut off for the calculated fold change was set to -1.5 and 1.5. (negative fold change values imply downregulation of the gene after bedrest, positive fold changes mean upregulation of the gene after bedrest). No genes did meet the cut of criteria.

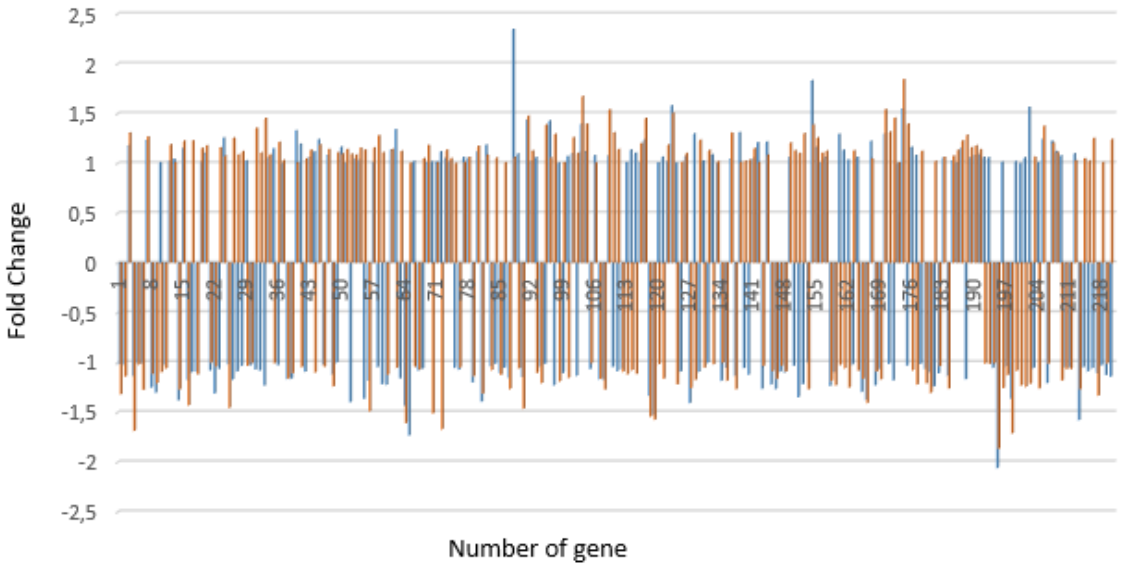

Figure 10 Fold change values of 220 genes coding for muscle proteins from are depicted. Blue codes for genes of the bedrest group, orange codes for the bedrest and exercise group

The following table shows the 12 significant changed genes from the data of the study group bedrest + exercise.

Molecules	
Fold Change up-regulated	**Exp.Value**
HSPB3	1.841
YAP1	1.673
IGF1	1.541
NOV	1.539
TRIM63	1.504
Fold Change down-regulated	
GDAP1	-1.866
FBXO32	-1.709
IGF1	-1.686
SGCB	-1.671
NOS1	-1.606
KCNH2	-1.575
PRSS12	-1.512

Figure 11

8 genes KCNH2, FBXO32, IGF1, YAP1, PRSS12, SGCB, NOS1 and TRIM63 have been recognized via the diseases and functions tool of IPA, which are involved in / inducing muscular changes. A canonical diagram (see figure 13) displays how these genes affect the skeletal

muscle. KCNH2, NOS1, HSPB3, GDAP1 and TRIM63 are expressed genes that are in common between the bedrest and bedrest and exercise group

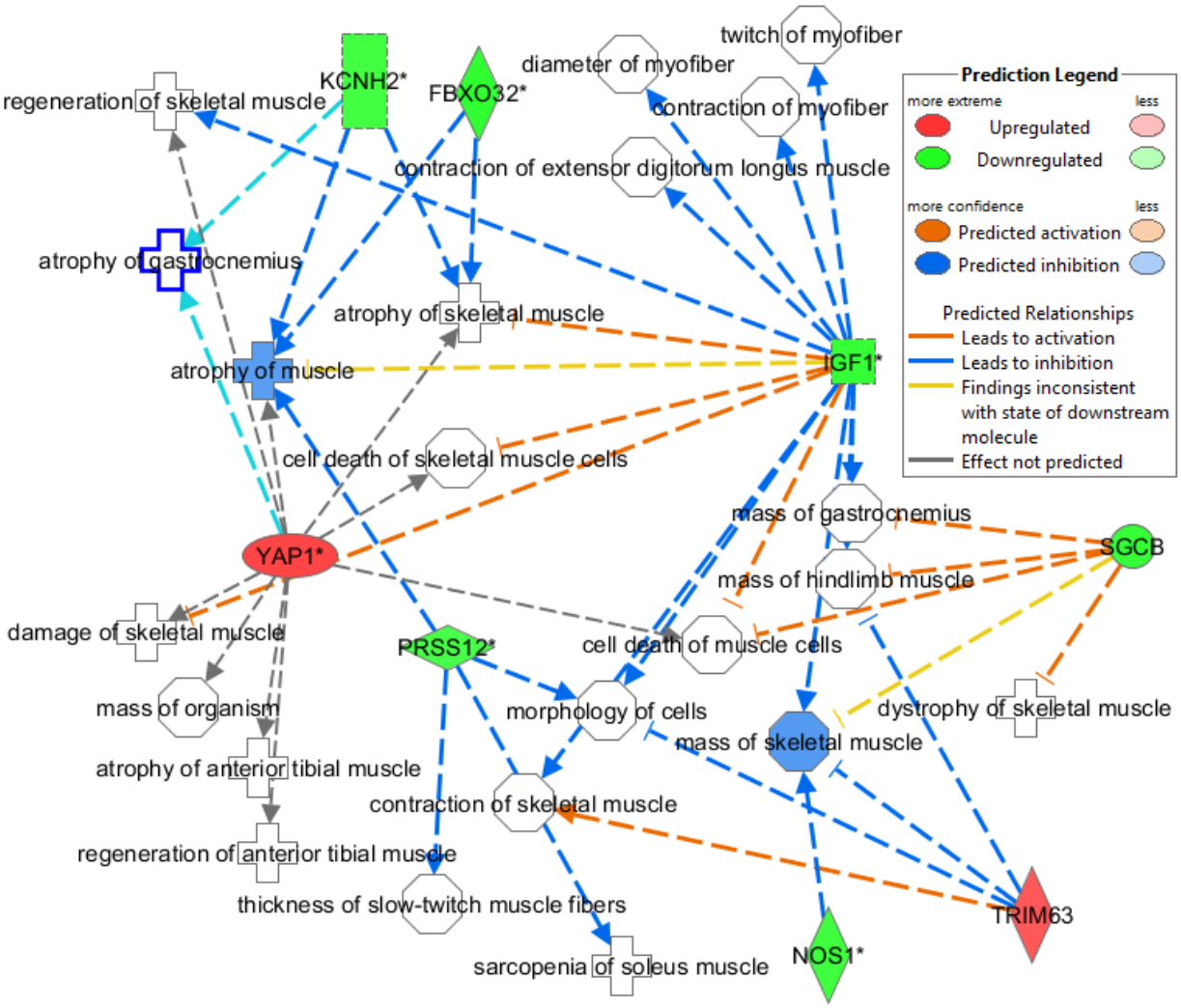

Figure 12 This diagram shows the influence of the up/downregulated genes (KCNH2, FBXO32, IGF1, YAP1, PRSS12, SGCB, NOS1 and TRIM63) on the skeletal muscle.

This figure was obtained from IPA.

5 Discussion

We are the first to explore the effects of bedrest at a molecular level using Transcriptomics. Our findings show that bedrest has an effect on gene expression. The findings negatively answer the question “Does exercise prevent muscle loss”. No gene was found that significantly changed between the group bedrest and bedrest and exercise. Comparing the two study groups, five genes overlap. They all have the same expression polarity.

The first gene encodes for the Homo sapiens potassium voltage-gated channel, subfamily H, member 2 (KCNH2). It is downregulated having the same expression polarity in bedrest and bedrest + exercise. Wang X. et al have showed that in healthy mice an increase of the channel amount induces atrophy by activating the ubiquitin protease pathway and a decrease suppresses atrophy (98). According to IPA, dominant negative KCNH2 mutant mice have less atrophy of gastrocnemius and skeletal muscle. KCNH2 expression is linked to skeletal muscle atrophy, its down regulation inhibits bedrest associated atrophy. As KCNH2 is down regulated (see figure 12) it should inhibit skeletal muscle atrophy.

Homo sapiens nitric oxide synthase 1 (NOS1) is downregulated as well and has the same expression polarity in bedrest and bedrest and exercise. The gene codes for nNOSμ, a heart and skeletal muscle specific protein which synthesizes nitric oxide (NO). Nitric oxide synthases (NOS) are expressed almost everywhere in the body, with high concentrations in the skeletal muscle. The muscular NOS produces NO, which plays a major role in signaling pathways regulating the structural and functional adaption in skeletal muscles. E.g. the regeneration of skeletal muscle cells, force production (E-C coupling), regulation of muscular contraction, allocation of glucose and differentiation of myocytes (99). Wang M. et al. showed in a rat model, that after 22 days of NOS inhibition, the muscle mass and the muscle fiber cross-sectional area decreased by 40% (100). IPA attributes NOS1 a positive effect on skeletal muscle mass (figure 12). As NOS1 is down regulated it should lead to a decline in skeletal muscle mass.

Homo sapiens tripartite motif containing 63 (TRIM63), also called MURF1, codes for the iris and skeletal muscle located E3 ubiquitin ligase. It has the same expression polarity in bedrest and bedrest and exercise. Its main function is the degradation of myosin heavy and light chains, the muscular creatinine kinase and myosin binding protein. Files et al. showed in a mouse study

that by inhibiting TRIM63 the effect of the gene was abolished, while the control group developed atrophy of skeletal muscle with loosing 25% of the muscle fiber diameter (101). According to figure 12 TRIM63 has a decreasing effect on skeletal muscle mass and the cell morphology leading to a decline in both macroscopic and cellular levels. But TRIM63 also has a positive effect on skeletal muscle contraction strength. TRIM63 was up regulated, meaning that skeletal muscle mass should have declined and skeletal muscle contraction strength should have increased in the probates.

Homo sapiens heat shock 27kDa protein 3 (HSPB3) encodes a muscle specific small heat shock protein. It has the same expression polarity in bedrest and bedrest and exercise. Autosomal dominant distal hereditary motor neuropathy type 2C is caused by a mutation of HSPB3 (102) and mutations of HSPB3 are associated with certain neurodegenerative diseases (103) and lead to congenital myopathy (104). HSPB3 binds to HSPB2 during muscle differentiation, this complex is important for muscle maintenance. HSPB3 was up regulated, meaning that is may inhibit skeletal muscle atrophy, but little is known about the functions of the gene.

Homo sapiens ganglioside-induced differentiation-association protein 1 (GDAP1), has the same expression polarity in bedrest and bedrest and exercise. No connection between the gene and skeletal muscle was found. During neuronal development GDAP1 might be needed (105). Charcot-Marie-Tooth Disease Type is associated with a GDAP1 mutation (106). This disease leads finally to muscular atrophy but this has no connection to bedrest associated muscle atrophy.

Limitations

It is possible that our results are limited in application as the sample size is limited. However, we are able to detect that transcriptomics can be used to assess changes at the molecular level during immobilization, with and without exercise. Future studies are required to confirm these changes using larger sample size.

Another limitation of this study is that we only examined the changes pre- and post-bedrest. It is possible that examining these two time points does not sufficiently allow us to follow the time course of the changes (e.g. what happens during the first week of immobilization, etc.). In the

future multiple serial samplings should be done to assess the time course of changes at the protein level.

The participants in this study do not represent the average elderly patient on a typical internal medicine ward. Nevertheless the underlying pathophysiological mechanisms might be the same.

Conclusions and clinical application

Five genes change equally in the two study groups KCNH2, NOS1, HSPB3, GDAP1 and TRIM63. NOS1 and TRIM63 might have a negative effect on skeletal muscle mass, whereas KCNH2 and HSPB3 might inhibit muscle atrophy. They could be part of a system which prevent excessive muscle loss during immobilization.

NOS1 and TRIM63 seem to have a higher influence on muscular atrophy. A clinical approach to muscle atrophy during immobilization might be to find interventions or medications that up regulate NOS1 expression and down regulate TRIM63 expression in patients with medical treatment.

It also appears that at transcriptomics level exercise intervention during bedrest does not seem to show a positive effect. An explanation of this could be embedded in the exercise training itself. For example, the effect of WBV training on the musculoskeletal system is not yet clear. Various studies have shown that WBV training is linked to an increase maximum strength and muscular power (36,107), whereas other study findings suggest that WBV training compared to the same exercises without WBV has only low effect on muscular strength (37,38). It is possible that the ideal training settings and devices to induce maximum effects on the musculoskeletal are not well defined (38). More studies are needed to evaluate the effects of WBV training as well as to identify the optimal training level and duration.

In summary, transcriptomics can be used to study changes at the molecular level. As this is a rapidly evolving field, especially the OMICs field, future studies should explore this area in greater depths so that the mechanistic aspects of immobilization and counter-measures become clear. Indeed, transcriptomics and metabolomics may show changes long before they affect physiological systems and consequently be used to prevent changes or at least to alleviate them well in advance of their occurrence.

6 List of abbreviations

- F-actin	Filamentous actin
- G-actin	Globular actin
- ATP	Adenosine triphosphate
- ADP	Adenosine diphosphate
- P	Phosphate
- Ca	Calcium
- Ach	Acetylcholine
- E-C coupling	Excitation-contraction coupling
- T-tubule	Transverse tubule
- DHPR	Dihydropyridin receptor
- MHC	Myosin heavy chain
- e.g.	Exempli gratia
- mRNA	Messenger ribonucleic acid
- cRNA	Complementary ribonucleic acid
- RNA	Ribonucleic acid
- NO	Nitrogen monoxide
- PGI_2	Prostacyclin
- BMD	Body mass index
- AIDS	Acquired immunodeficiency syndrome
- COPD	Chronic obstructive pulmonary disease
- SF	Spaceflight
- CSA	Cross-sectional area
- IGF-1	Insulin-like growth factor 1
- PI3K	Phosphatidylinositol 3 kinase
- mTOR	Mechanistic Target of Rapamycin
- AKT	Protein kinase B
- SMAD 2/3/4	Mothers against decapentaplegic homolog 2/3/4
- FoxO3	Forkhead box O3
- NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
- Myostatin	Growth-differentiation factor 8

- MyoD	Myogen factor 3
- TGFβ	Transforming growth factor beta
- ACVR2/ACVR2B	Activin receptor 2
- ALK4/5	Activin receptor-like kinase 4 /5
- N-end	The start of a protein/polypeptide terminated by an amino acid with a free amine group
- MuRF1	Muscle-specific RING Finger protein1
- Ub	Ubiquitin
- Ub E2	Ub carrier protein
- CMA	Chaperone-mediated autophagy
- PINK1	PTEN-induced putative kinase 1
- TNFα	Tumor necrosis factor alpha
- IκB	Nuclear factor of kappa light polypeptide gene enhancer
- IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
- BH-FDR	Benjamini-Hochberg false discovery rate
- TWEAK	TNF-related weak inducer of apoptosis
- GO	Gene Ontology
- Ca	Calcium
- ESA	European space agency
- SBP	Systolic blood pressure
- DBP	Diastolic blood pressure
- HR	Heart rate
- VO_2 max test	Maximal oxygen consumption
- CBC	Cell blood count
- HPG	Human genome project
- CNVs	DNA Copy number variations
- SNPs	Single nucleotide polymorphisms
- GWAS	Genome-wide association studies
- WBV	Whole body vibration
- IPA	Ingenuity Pathway Analysis

- KCNH2 Homo sapiens potassium voltage-gated channel, subfamily H, member 2
- NOS1 Homo sapiens nitric oxide synthase 1
- NO nitric oxide
- NOS Nitric oxide synthases

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8 Figures