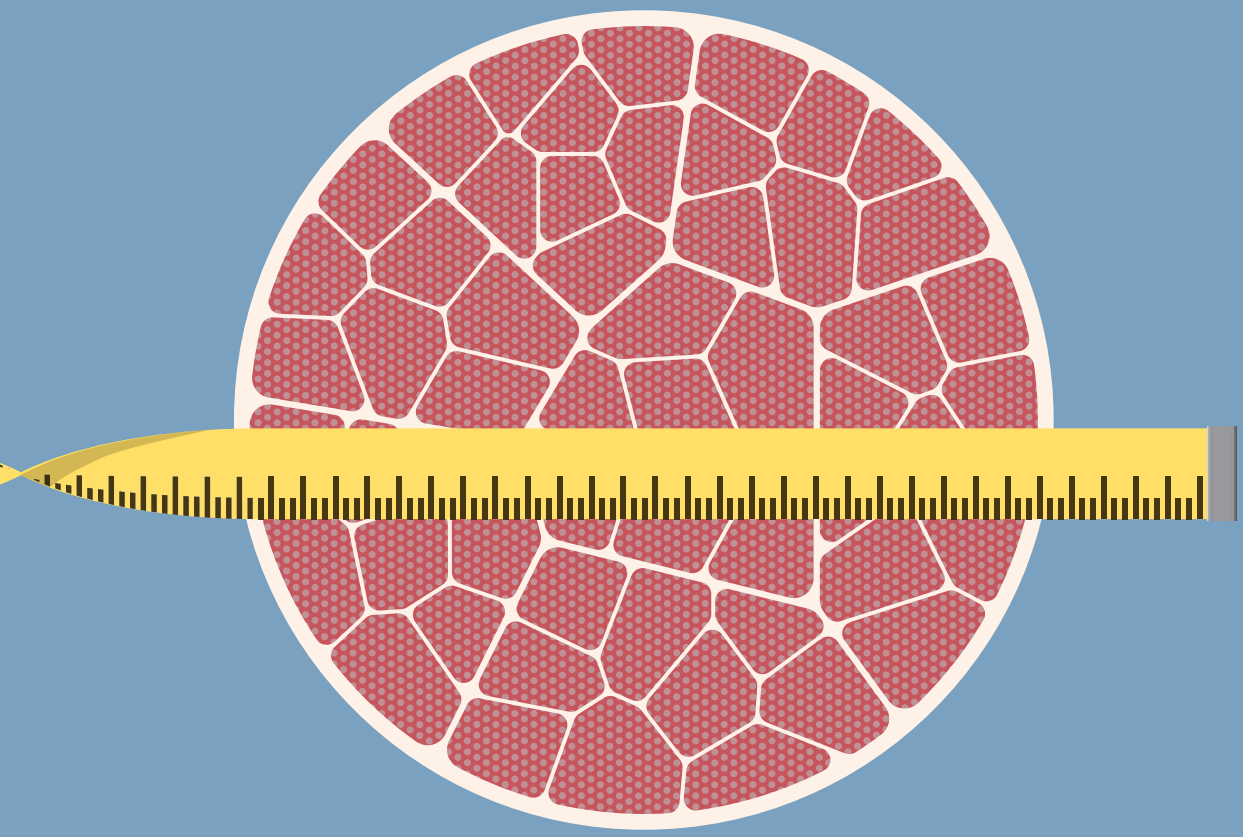
Molecular Assessment of Muscle Health and Function

The effect of age, nutrition and physical activity on the human muscle transcriptome and metabolome



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Roland W. J.Hangelbroek

Thesis

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Chapter 1 : General Introduction

'The idea is to die young as late as possible.' - Ashley Montagu

Healthy aging

Advances in medicine and our understanding of human biology have led to an increase in longevity, while the rise of contraceptives has led to a decrease in fertility rates. These two factors will lead to an increased proportion of older adults in the world population [1]. While longevity is in itself incredibly valuable, increased age is unfortunately often associated with health problems. These health problems consist primarily of non-communicable chronic diseases, including type 2 diabetes, disability, cancer, cardiovascular disease and cognitive decline. As a consequence, the increase in life expectancy is expected to lead to increased costs of health care, while the population that can support these health care costs is expected to diminish [2].

An important strategy to deal with population aging has been to promote healthy aging; not only to prevent mounting health care costs, but also to maintain independence and quality of life of older populations for as long as possible. Aging is thought to be caused by a gradual accumulation of molecular and cellular damage [3]. However, aging is not necessarily a steady decline towards impaired physiological performance due to this damage, but rather a gradual decline in the ability to handle stressors and decreased reserves to handle cellular and molecular damage [4]. These stressors include for example smoking, a large high fat meal, but also physical activity or lack thereof. The healthy aging phenotype can be described as not only the absence of disease, but also a preserved ability to handle stressors [4]. While healthy aging is partly beyond our control due to for example genetic traits, maintaining a healthy lifestyle over the life course, specifically with regard to nutrition and physical activity, is a key component of healthy aging [5, 6].

Frailty and Sarcopenia

A phenotype that is close to the opposite of the healthy aging phenotype is the frail phenotype [4]. Frailty is characterised by a decreased resistance to stressors, leading to increased risk of adverse health outcomes, and is prevalent (~9.6–10.2%) among older populations [7, 8]. Fried *et al*. defined five characteristics for frailty: unintended weight loss, exhaustion, weakness, slow gait speed and low physical activity [9]. While frailty encompasses a wide variety of factors, a major component of (physical) frailty is sarcopenia: age-related loss of muscle mass. Sarcopenia and frailty overlap; generally frail individuals exhibit sarcopenia and sarcopenic individuals are often frail [10]. A related term to sarcopenia is dynapenia, the age-related loss of muscle strength [11].

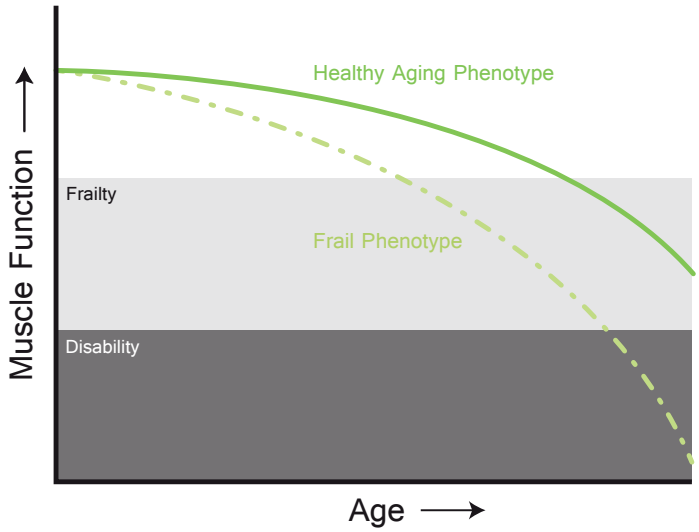

Figure 1.1 – Trajectories of muscle function in the frail and healthy ageing phenotypes.

Decreased muscle size and strength, as is the case with physical frailty and sarcopenia, has been associated with a wide variety of negative health outcomes, including increased risk of hospitalization, physical disability and even death [12-14]. In figure 1.1 the trajectory associated with the healthy aging phenotype and the frail phenotype are contrasted with regard to muscle function. Although muscle mass and muscle strength are correlated, it has been argued that loss of muscle strength is a more important risk factor among older populations than muscle mass [11, 14]. Various factors are implicated in the cause of age-related loss of muscle mass and strength, highlighting the complexity of sarcopenia and dynapenia. Contributing factors that have been suggested include hormonal changes, physical inactivity, decreased satellite cell counts, insufficient energy and protein intake, vitamin D deficiency, shifts in muscle fibre type proportions, mitochondrial dysfunction, inflammation ('inflammaging') and decreased response to anabolic stimuli (anabolic resistance) [15-17].

Muscle atrophy

Repeated periods of muscle disuse due to hospitalization and disease (bed rest) or bone fractures (limb immobilization) can also significantly contribute to frailty, sarcopenia and dynapenia. Short periods of muscle disuse can lead to rapid losses of muscle mass and strength [18]. For example, five days of knee immobilization among young healthy volunteers led to a drop of ~3.5% in muscle cross-sectional area and a ~8.5% drop in muscle strength

[19]. Regaining these losses can take considerable time, especially among older individuals who have a decreased capacity to regain these losses [20-22]. Over the lifetime these losses of muscle mass and strength can accumulate, particularly among older individuals [23]. Limiting the losses during muscle disuse is of vital importance to manage the aging trajectory of muscle function and to maintain muscle health in later stages of life (figure 1.2).

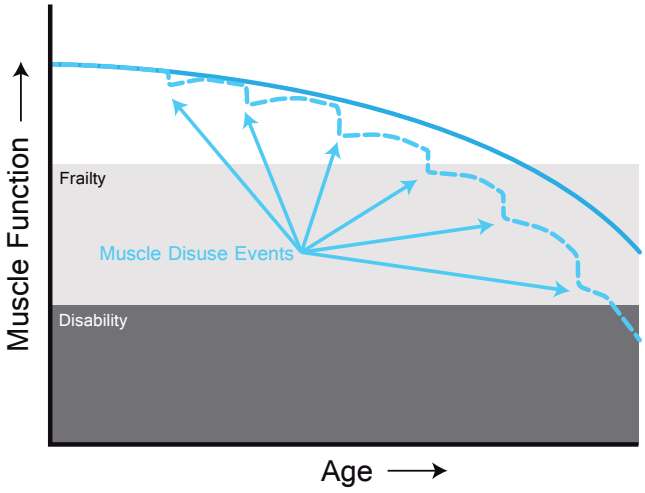

Figure 1.2 – Trajectories of muscle function with or without muscle disuse events

Muscle atrophy occurs when there is an imbalance in muscle protein synthesis and muscle protein breakdown. In the case of anabolic resistance, mentioned previously, the anabolic response to dietary protein is diminished, leading to a net loss in muscle mass over longer periods of time [17]. By contrast, cancer cachexia, the wasting of skeletal muscle due to cancer, can lead to a dramatic increase in muscle protein breakdown [24]. Due to the difficulty of measuring the balance between muscle protein synthesis and breakdown over prolonged periods of time, some discussion exists which is dominant in muscle atrophy due to disuse [25]. Potential mechanisms for muscle protein breakdown include autophagy, apoptosis, calpains and ubiquitin mediated proteolysis [26, 27]. Of these mechanisms, ubiquitin mediated proteolysis has been the most studied in skeletal muscle. However, each of these mechanisms may be involved in muscle atrophy and degree of their involvement likely depends on the underlying cause of muscle loss.

Preventing muscle loss

Various strategies have been proposed to attenuate muscle losses due to disuse and age. Strategies that are effective in enhancing muscle performance and size in athletes may be especially useful as countermeasures. Resistance-type exercise training is one of the most effective ways of enhancing muscle mass and strength among older populations [28-30]. The beneficial effects of resistance-type exercise training are, in general, enhanced when training is combined with increased energy and protein intake [31]. Unfortunately, resistance-type exercise training is in some cases not an option, such as for example when an individual is hospitalized or when a leg is fully immobilized due to a fracture. In these cases, other measures are required to prevent muscle losses. Other possible interventions to prevent muscle loss include pharmacological interventions, such as anabolic androgenic steroids and selective androgen receptor modulators, as well as stimulating muscle contractions using neuromuscular electrical stimulation [32].

Possible nutritional strategies to combat muscle loss are protein supplementation and supplementation with bioactive compounds (for instance creatine, leucine, β-Hydroxy β-Methylbutyrate and ursolic acid) [33, 34]. Undernutrition among older adults is also associated with decreased muscle mass and strength [35]. Vitamin D deficiency in particular has been studied as a contributing factor to age-related muscle loss. Several observational studies have found a link between vitamin D status and muscle function [36-40]. However, it is still unclear how vitamin D affects muscle function [41]. In the context of muscle loss due to muscle disuse, creatine supplementation has shown promise either by attenuating muscle loss during immobilization or by enhancing muscle gain during resistance-type exercise training after knee immobilization [42, 43].

Transcriptomics and metabolomics

Age, nutrition and exercise can have a wide range of effects in skeletal muscle at the molecular level. Exercise alone can affect various pathways and metabolic processes in the muscle, depending on the type, intensity and duration of the exercise [44, 45]. Similar heterogeneity can be observed in the processes affected by aging, nutrition and muscle atrophy. The use of high throughput ~omics techniques allows for simultaneous investigation of the various processes involved by measuring a large amount of genes, proteins or metabolites simultaneously. Both transcriptomics and metabolomics will be employed in this thesis.

Previously, in the case of transcriptomics, activity specific genes were measured individually using qPCR or northern blots. Transcriptomic techniques, such as microarrays or RNA-seq, measure approximately mRNA of 20.000 human genes in a single sample. Measuring the transcriptional activity of (almost) all known genes not only allows for quantification of familiar genes, but also to measure the activity of genes not previously associated with a certain

biological process or stimulus. Combining sets of genes into pathways or genes known to be affected by a certain upstream regulator (such as a hormone, transcription factor or chemical) can shed light on the effect of an intervention or stimulus on larger cellular processes rather than individual genes. Transcriptomics has been used in the study of nutrition, exercise and pharmacological compounds and has especially proven invaluable in the study of the broad but subtle effects of nutrition, due to the maturity of the platforms [46, 47].

A newer development has been the field of metabolomics. Metabolomics primarily pertains to all small molecules and metabolites that occur in human metabolism [48]. These metabolites represent a fingerprint of cellular processes. The tissue metabolome is generally closer to the phenotype, allowing for the investigation of cellular processes in more detail, particularly energy metabolism, whereas the transcriptome represents the regulatory state of a cell or tissue [49]. The development and validation of metabolomics profiling of skeletal muscle allows for the application of this technology in in the context of muscle function [50].

Thesis outline

The effect of age, frailty and physical activity on the function and size of skeletal muscle is complex. A better understanding of the molecular mechanisms involved can provide new insights in potential strategies to maintain muscle function over the life course. This thesis aims to investigate these mechanisms and processes that underlie the effects of age, frailty and physical activity by leveraging the sensitivity and comprehensiveness of transcriptomics and metabolomics. Additionally, two potential nutritional strategies that can improve muscle function are investigated using transcriptomics.

In **chapter 2 and 3** the differences between frail older adults, healthy older adults and young individuals in the muscle metabolome and transcriptome are explored. Furthermore, the effects of resistance-type exercise on the muscle transcriptome and metabolome serve as 'benchmark' of a positive influence on muscle function. In **chapter 2** the focus is on the transcriptome, whereas in **chapter 3** the focus is placed on the metabolome. **Chapter 4** explores the effect of knee immobilization on the muscle transcriptome and metabolome. Creatine supplementation is evaluated as strategy to attenuate muscle losses during knee immobilization. **Chapter 5** investigates the effects of vitamin D (calcifediol) supplementation as potential strategy to improve muscle function among frail older adults. **Chapter 6** provides a comparison between knee immobilization and bed rest with regard to the muscle transcriptome. Furthermore, additional publically available datasets on physical inactivity models are explored and compared to identify a human signature of muscle atrophy. Various forms of muscle disuse are compared with the signature of frailty (described in **chapter 2**) to identify a potential surrogate model for age-related muscle loss. Lastly, **chapter 7** provides an overall discussion of the results presented in this thesis, as well as future perspectives.

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Chapter 2 - Expression of protocadherin gamma in skeletal muscle tissue is associated with age and muscle weakness

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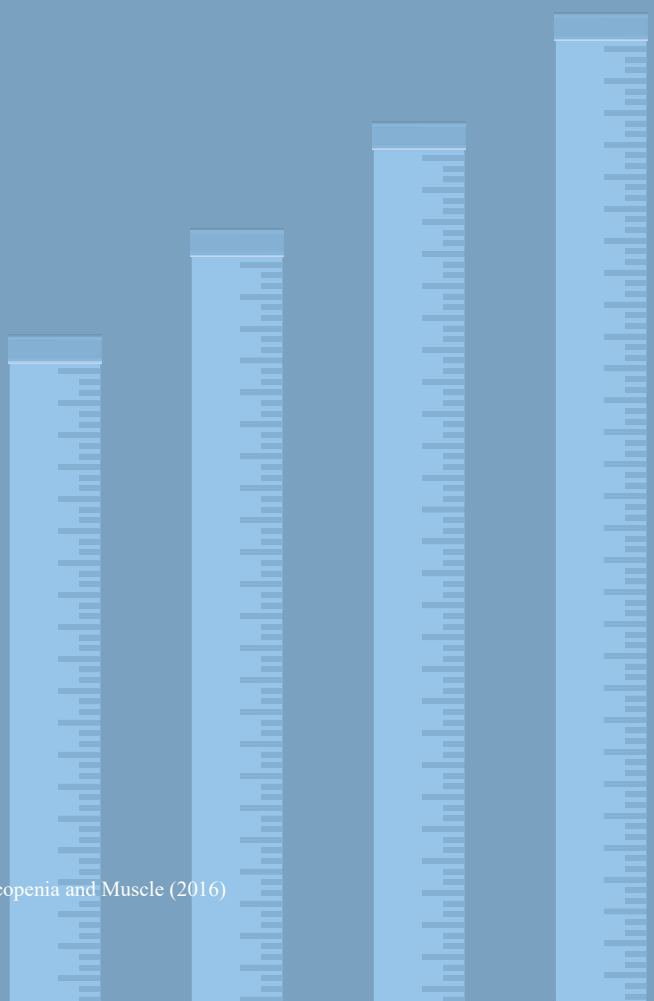

Abstract

The skeletal muscle system plays an important role in the independence of older adults. In this study we examine differences in the skeletal muscle transcriptome between healthy young and older subjects and (pre-)frail older adults. Additionally, we examine the effect of resistance-type exercise training on the muscle transcriptome in healthy older subjects and (pre-)frail older adults.

Baseline transcriptome profiles were measured in muscle biopsies collected from 53 young, 73 healthy older subjects and 61 frail older subjects. Follow-up samples from these frail older subjects (31 samples) and healthy older subjects (41 samples) were collected after 6 months of progressive resistance-type exercise training. Frail older subjects trained twice per week and the healthy older subjects trained three times per week.

At baseline genes related to mitochondrial function and energy metabolism were differentially expressed between older and young subjects, as well as between healthy and frail older subjects. 307 genes were differentially expressed after training in both groups. Training affected expression levels of genes related to extracellular matrix, glucose metabolism and vascularization. Expression of genes that were modulated by exercise training were indicative of muscle strength at baseline. Genes that strongly correlated with strength belonged to the protocadherin gamma gene cluster (r=-0.73).

Our data suggest significant remaining plasticity of ageing skeletal muscle to adapt to resistance-type exercise training. Some age-related changes in skeletal muscle gene expression appear to be partially reversed by prolonged resistance-type exercise training. The protocadherin gamma gene cluster may be related to muscle denervation and re-innervation in ageing muscle.

Introduction

The number of people aged above 65 has increased rapidly over the past few decades, and is likely to increase progressively [1]. Because senescence is associated with a wide range of afflictions, including physical disability, cancer, heart disease and diabetes, the demand for care for older people will further increase. The loss of skeletal muscle mass and function with aging leads to frailty and results in the loss of independence of older adults.

Frailty, and related sarcopenia, are very complex and many factors contribute to their aetiology. This includes physical inactivity, malnutrition, hormonal changes and changes within the muscle [2-4]. Mitochondrial function decreases with age [5], fast-twitch muscle fibres demonstrate a smaller cross-sectional area [6], protein synthesis capacity is reduced [7], anabolic signals are less effective [8, 4], and there are fewer satellite cells to regulate adaptive responses to stimuli [9]. Muscle cells of older adults can also undergo continuous cycles of denervation and reinnervation, which can lead to both weakness and loss of muscle mass [10, 11].

One of the most effective strategies to improve muscle mass and strength in adults is physical exercise [9, 12]. Resistance-type exercise is particularly suitable to curtail muscle loss and muscle weakness in older people. In accordance, quality of life is improved after participating in resistance-type exercise training [13]. Some even claim that resistance-type exercise training reverses ageing in skeletal muscle [14].

To elucidate some of these complex processes that occur in skeletal muscle during ageing, we examined the effects of prolonged resistance-type exercise training in frail and healthy older subjects on the skeletal muscle transcriptome. By comparing genome-level gene expression in frail and pre-frail older subjects, healthy older subjects and young subjects we aim to better understand the molecular causes of frailty. Secondly, we aimed to determine the effect of resistance-type exercise training on the skeletal muscle transcriptome in both frail and healthy older people.

Methods

Experimental Design

We collected a total of 259 muscle biopsy samples from pre-frail and frail older subjects (61 subjects, 92 samples), healthy older subjects (73 subjects, 114 samples) and young males (53 subjects, 53 samples). Some of these samples were follow-up samples taken after 24 weeks of resistance-type exercise training (31 samples from the frail older subjects, 41 samples from the healthy older subjects). Training for both groups was similar and consisted of progressive full-body resistance-type exercise training. However, the frail older group had training sessions twice per week, whereas the healthy older group trained three times per week. In addition, subjects took a protein or control drink for the duration of the study. The healthy older group received a 15 gram portion of milk protein or control supplement at breakfast. The frail older group received a similar drink containing 15 gram supplement drink (milk protein or control) at breakfast and lunch. More details can be found in the respective papers [15, 16]. Table 2.1 shows the characteristics of our study population at baseline. Table 2.2 shows the effect of the training intervention on the older subjects that were included in this study and where follow-up data is available.

Table 2.1 – Subject characteristics of the baseline only subjects. FE = frail older subjects, HE = healthy older subjects, YO = young male subjects.

	FE	HE	YO
N (male / female)	24 / 6	27 / 5	53 / 0
Age (years)	79.8 ± 8.9	74.1 ± 4.5	21.3 ± 2.4
Height (m)	1.71 ± 0.09	1.73 ± 0.08	1.84 ± 0.06
Weight (kg)	80 ± 12.4	75.9 ± 12.9	76.5 ± 10.3
BMI (kg / m^2)	27.3 ± 4.2	25.2 ± 3.2	22.6 ± 3
Body Fat (%)	28.8 ± 7.2	23.4 ± 5.5	15.4 ± 4.6
Lean Mass (kg)	52.1 ± 6.3	55.5 ± 8.6	61.9 ± 6
Leg Extension 1RM (kg)	65 ± 20	68 ± 17	124 ± 20
Leg Press 1RM (kg)	127 ± 31	155 ± 41	203 ± 36

Subjects

Biopsies from frail and pre-frail older subjects were collected from participants of two studies performed by Tieland *et al.* [15, 17]. For these studies frail and pre-frail older subjects were selected based on the Fried criteria for frailty [2]. These subjects will hereafter be referred to as frail older subjects. These characteristics are unintentional weight loss, weakness, self-reported exhaustion, slow walking speed, and low physical activity. Subjects in the healthy older group were not considered frail by any of these criteria at the start of the intervention

study [16]. Several additional baseline samples from healthy older subjects were collected from several studies from our group [18, 19]. These samples were taken before any intervention was undertaken and serve as additional reference samples. Baseline samples from young subjects were from healthy male subjects [20]. These were also taken before any intervention took place and serve as reference samples. All studies were approved by the medical ethical committee of either Wageningen University or Maastricht University and comply with the Declaration of Helsinki.

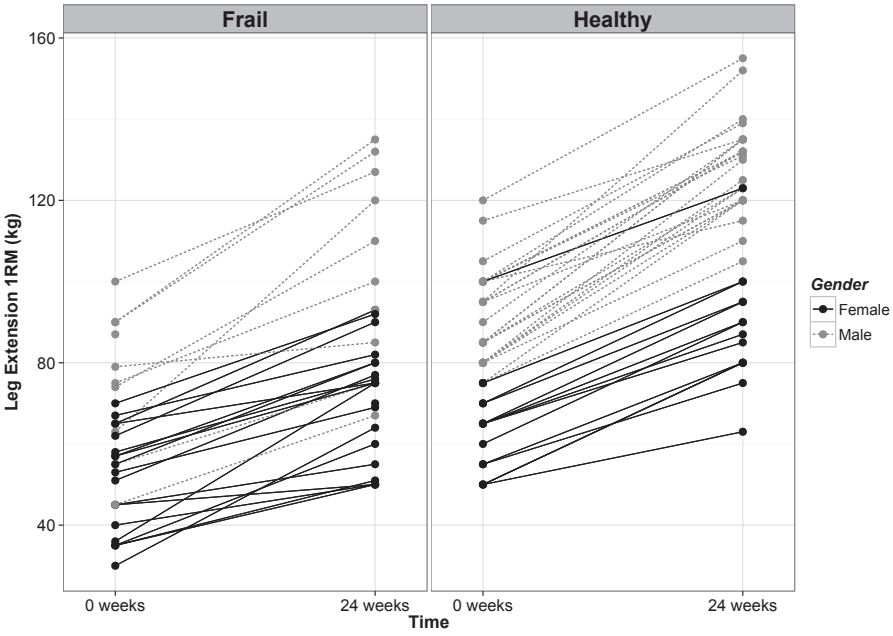

Figure 2.1 A and B – Change of leg extension 1RM after prolonged exercise training for each individual. Left are frail elderly subjects, right are healthy elderly subjects.

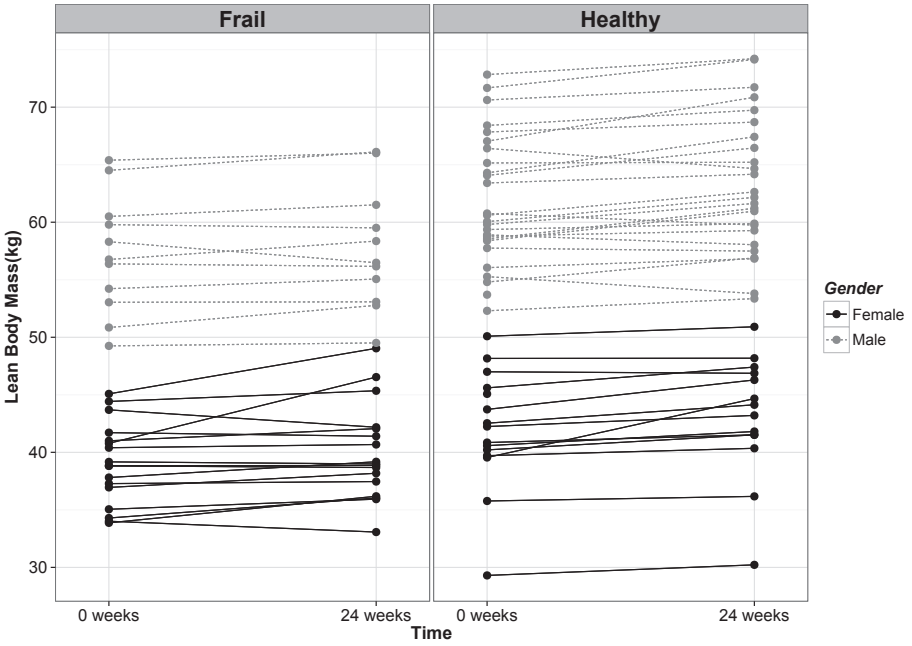

Figure 2.2 A and B – Change of lean body mass after prolonged exercise training for each individual. Left are frail older subjects, right are healthy older subjects.

Table 2.2 – Subject characteristics of the subjects with before and after samples. FE = frail older subjects, HE = healthy older subjects, YO = young male subjects. * indicates a significant effect of resistance-type exercise training ($p < 0.05$).

	FE pre	FE post	HE pre	0HE post
N (male / female)	11 / 20		26 / 15	
Age (years)	76.5 ± 7.0		69.9 ± 5.0	
Height (m)	1.66 ± 0.09		1.71 ± 0.09	
Weight (kg)	78.5 ± 13.6	79.6 ± 14.1*	76.7 ± 13.2	77.1 ± 13.2
BMI (kg / m²)	28.5 ± 4.1	29 ± 4.3 *	26.1 ± 2.8	26.2 ± 2.8
Body Fat (%)	36.7 ± 8.5	36.6 ± 8.8	25.9 ± 5.9	24.4 ± 5.9 *
Lean Mass (kg)	46.1 ± 10.0	46.9 ± 9.9 *	54.6 ± 11.1	56.0 ± 11.3 *
Leg Extension 1RM (kg)	59 ± 18	81 ± 24 *	81 ± 17	114 ± 23 *
Leg Press 1RM (kg)	130 ± 35	178 ± 49 *	179 ± 40	230 ± 50 *

Muscle biopsy

Muscle samples were obtained with a 5 mm Bergstrom muscle biopsy needle from the m. vastus lateralis, after local anaesthesia of the skin and fascia. Samples were freed from any visible blood and non-muscle tissue and immediately frozen in liquid nitrogen and then stored at -80° C. All samples were obtained in the morning, in an overnight fasted state, with at least 3 days of no heavy physical activity.

Sample preparation and microarray analysis

Total RNA was isolated from the skeletal muscle tissue by using Trizol reagent (Invitrogen, Breda, Netherlands). Thereafter RNA was purified using the Qiagen RNeasy Micro kit (Qiagen, Venlo, Netherlands) and RNA quality was checked using an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, Netherlands). Total RNA (100 ng) was labelled using an Ambion WT expression kit (Life Technologies, Bleiswijk, Netherlands) and hybridized to human whole genome Genechip Human Gene 1.1 ST arrays coding 19.732 genes, (Affymetrix, Santa Clara, CA). Sample labelling, hybridization to chips and image scanning was performed according manufacturer's instructions.

Data analysis

Microarray signals were normalized using robust multichip average (RMA). Data was filtered using Universal exPression Codes filtering (UPC) with a 50% cut-off, corresponding to a 50% likelihood that a gene is expressed [21]. Significant differences of individual genes were tested using the limma R library [22]. Baseline differences were tested between the three groups (frail older, healthy older or young) . Our model included gender, supplementation and group. For the effect of exercise we included subject, gender, time and supplementation in the model. The training effect for frail older and healthy older subjects was analysed separately due to differences in training frequency. P-values were adjusted using false discovery rate (FDR) [23]. A q-value below 0.05 was considered significant. Pathway analyses were performed using Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) on the filtered dataset with the UPC filtered genes used as the background. A sparse partial least squares (sPLS) model for leg extension 1RM was made using the caret R library [24]. The dataset was split into a training set (75%) and a testing set (25%) before fitting the model using cross-validation. This model was validated using 10 times repeated 10-fold cross-validation. Final number of components for the sPLS model selected by grid search was 3. Principal Component Analysis (PCA) was performed using the FactoMineR R library [25]. Plots were made using the R libraries ggplot2 and gplots [26, 27].

Results

Baseline differences in transcriptome

Large differences in gene expression profiles between young and older adults (healthy and frail) were found, with the expression of 5228 genes significantly differentially expressed between young subjects and both groups of older subjects. However, fold changes of the majority of these genes were relatively subtle, which suggested small but consistent differences between these groups. Only 825 genes out of these 5228 genes showed fold changes higher than 1.2. Venn-diagrams can be found in supplementary figures S2.1A and S2. 1B. The top 20 genes that were significantly different at baseline between the three groups are presented in table 2.3. Top canonical pathways reported by IPA include oxidative phosphorylation, TCA cycle and glucose metabolism (supplementary figure S2.2).

Table 2.3 – Top 20 genes significantly different between young and old. FE = frail older subjects, HE = healthy older subjects, YO = young men.

Gene	FC FE vs. HE	FC FE vs. YO	FC HE vs. YO	q-value FE vs. HE	q-value FE vs. YO	q-0alue HE vs. YO
IGFN1	-1.12	-6.43	-5.71	0.64	0.00	0.00
UNC13C	1.07	6.20	5.78	0.60	0.00	0.00
MYLK4	-1.35	-5.13	-3.81	0.07	0.00	0.00
C12orf75	1.45	4.54	3.13	0.01	0.00	0.00
SLC38A1	-1.11	-3.50	-3.14	0.55	0.00	0.00
HCN1	1.16	3.36	2.90	0.30	0.00	0.00
MYH8	1.16	3.28	2.83	0.50	0.00	0.00
CFAP61	1.36	3.39	2.49	0.00	0.00	0.00
NR4A3	-1.86	-3.70	-2.00	0.02	0.00	0.00
FAM83B	1.11	2.91	2.63	0.39	0.00	0.00
DAAM2	-1.06	-2.51	-2.36	0.42	0.00	0.00
NNMT	1.33	2.71	2.04	0.03	0.00	0.00
ZNF382	1.21	-2.09	-2.54	0.05	0.00	0.00
TPPP3	1.33	2.62	1.97	0.01	0.00	0.00
COL28A1	1.07	2.36	2.20	0.42	0.00	0.00
METTL21EP	1.05	-2.19	-2.29	0.78	0.00	0.00
HIST1H3E	1.11	-2.08	-2.30	0.30	0.00	0.00
SNORD115-32	1.02	2.17	2.14	0.91	0.00	0.00
SERPINA5	1.04	-2.11	-2.19	0.89	0.00	0.00
METTL21C	1.98	2.84	1.44	0.00	0.00	0.03

Table 2.4 shows the top 20 genes that were different between healthy and frail older subjects at baseline. Top genes included METTL21C, FRZB and non-coding RNA. Pathways that were significantly different between the frail and healthy older subjects were related to glucose metabolism and RNA processing (supplementary figure S2.2). In general, expression of genes related to glucose metabolism were lower in both frail older and healthy older subjects compared to young, with frail older subjects showing the lowest expression of the groups. Principal Component Analysis (PCA) summarizes this observation, where the healthy older subjects seemed to be between the frail older subjects and the young subjects on the first two components (supplementary figure S2.3 and S2.4). While pathways related to mitochondrial function were some of the most significantly affected pathways, the fold changes of the individual mitochondrial genes were relatively small. Fold changes for these mitochondrial genes were in the range of 1.1 and 1.2.

Table 2.4 – Top 20 genes significantly different between the frail elderly and the healthy elderly at baseline. FE = frail older subjects, HE = healthy older subjects.

Gene	FC FE vs. HE	q-value FE vs. HE
METTL21C	1.98	0.00
NR4A3	-1.86	0.02
VTRNA1-1	-1.71	0.00
MIR206	1.58	0.00
SNORA38B	-1.58	0.00
S100A8	1.51	0.04
FRZB	-1.48	0.03
HES1	-1.47	0.00
P2RY13	1.46	0.00
MIR133B	1.45	0.01
C12orf75	1.45	0.01
SNORA60	-1.44	0.00
SNORD60	-1.43	0.00
LYZ	1.43	0.03
SNORD80	-1.42	0.00
SNORD82	-1.42	0.00
SNORD29	-1.41	0.00
EVI2B	1.40	0.01
UPK3A	-1.40	0.00
ID1	-1.40	0.00

Effect of prolonged resistance–type exercise training

Prolonged resistance-type exercise training showed a significant effect on the gene expression profiles in both frail and healthy older people (431 and 1395 significantly changed genes, respectively). 307 genes were changed in both groups after resistance-type exercise training. Changes in expression of all these overlapping genes were in the same direction. A table with the top 20 genes changed by training is presented in table 2.5. Training resulted in the differential expression levels of many genes that are related to the connective tissue and the extracellular matrix, including collagen genes and laminin genes, suggesting significant tissue remodelling due to the training. Upstream analysis using IPA showed that TGF-β signalling-related genes were significantly activated in both groups, primarily due to the increased expression of collagen and laminin genes (supplementary figure S2.5). Other significant genes include myofibrillar proteins such as myosin heavy chain isoforms and troponin isoforms.

Genes related to glucose metabolism shifted away from the expression levels of the older subjects at baseline towards the levels of the younger phenotype. This trend is reflected in many other genes, where the majority of genes significantly changed by exercise training shifted towards 'younger' expression levels (325 genes out of 431 in the frail older subjects, 1106 out of 1395 in the healthy older subjects). Figure 2.3 shows a heat map of 184 genes that are significantly changed by training in both groups and are significant when comparing young with either frail or healthy older subjects. Most of these genes shift towards younger levels.

Table 2.5 – Top 20 genes significantly different in both frail elderly and healthy elderly subjects after training. FE = frail older subjects, HE = healthy older subjects.

Gene	FC FE training	FC HE training	q-value FE training	q-value HE training
FRZB	1.97	1.55	0.00	0.00
IGFN1	1.58	1.80	0.04	0.00
MYLK4	1.45	1.69	0.01	0.00
COL3A1	1.45	1.68	0.01	0.00
ANKRD2	-1.44	-1.61	0.01	0.00
THBS4	1.34	1.66	0.05	0.00
PFKFB3	1.61	1.38	0.01	0.01
COL4A1	1.35	1.46	0.00	0.00
CAPN6	1.37	1.45	0.03	0.00
COL1A2	1.35	1.45	0.03	0.00
EDNRB	1.24	1.56	0.01	0.00
GCNT2	-1.51	-1.28	0.00	0.00
CFAP61	-1.24	-1.54	0.03	0.00
C12orf75	-1.42	-1.33	0.01	0.00
CCDC80	1.34	1.40	0.03	0.00
OLFML2B	1.38	1.34	0.00	0.00
SPARC	1.28	1.44	0.00	0.00
COL4A2	1.32	1.37	0.00	0.00
LGI1	-1.31	-1.33	0.02	0.00
ACOT11	-1.30	-1.34	0.04	0.00

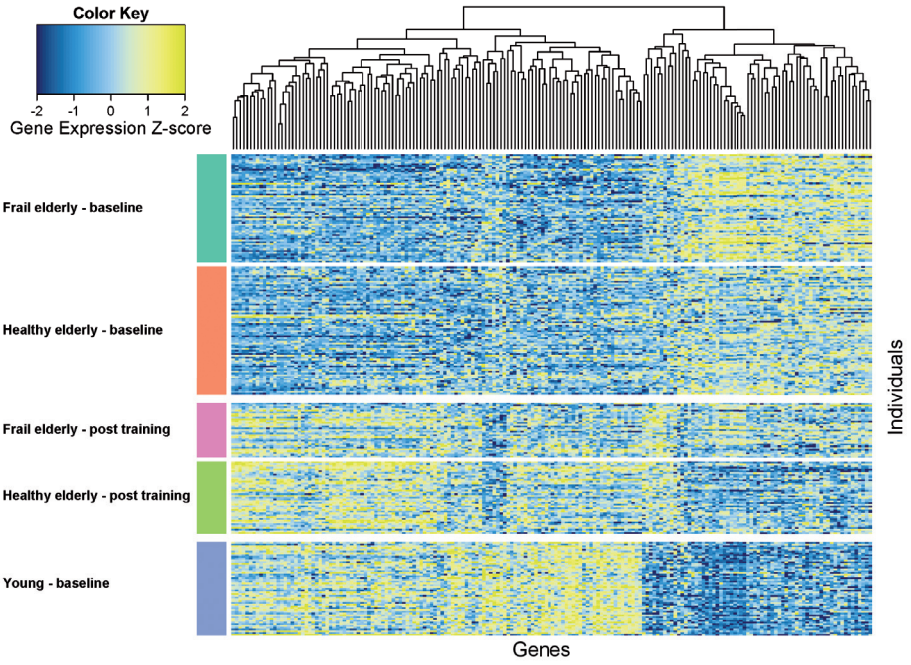

Figure 2.3 – heatmap of 184 genes that are significantly different between young and older subjects, and are significantly changed by prolonged resistance-type exercise training in both groups.

To further analyse the relationship between the 307 genes that are robustly after training in both groups changed (q-value < 0.05) we performed sPLS regression to calculate leg extension 1RM based on gene expression in the baseline samples. The aim was to evaluate whether differences in expression of the genes that were changed by training represent the overall strength of the muscle at baseline. The samples obtained after training were excluded for this analysis. A plot of the predicted leg extension 1RM strength against the measured leg extension 1RM strength is presented in figure 2.4. Gene importance for the final model is presented in table 2.6. Cross-validation mean R^2 of the model was 0.73, the mean RMSE was 17.7. The RMSE for the withheld testing set was 19.1. The top genes contributing to the model include genes from the protocadherin gamma gene cluster, CTNNBIP1, C20orf26 (CFAP61), C12orf75 and USP54. We calculated the eigengene for all protocadherin gamma genes and correlated this eigengene with leg extension 1RM. The protocadherin gamma eigengene showed a strong direct negative correlation (Pearson r = -0.73) with 1RM leg extension strength. A plot of this negative correlation is presented in supplementary figure S2.7.

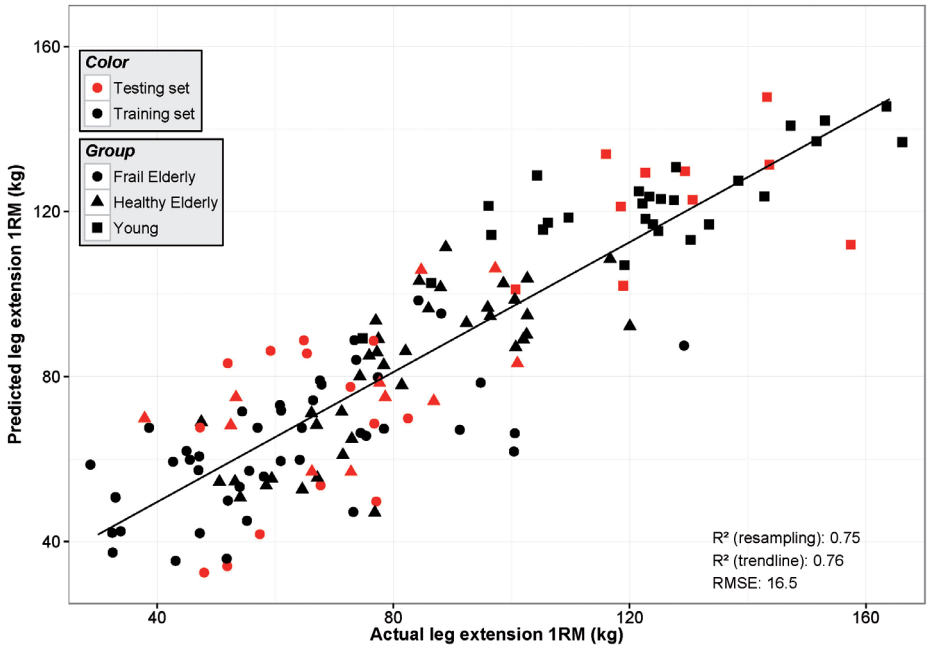

Figure 2.4 – Scatter plot of predicted leg extension 1RM of the baseline samples using sPLS and the actual measured 1RM. Red dots indicate samples that were part of the testing data set (25% of the total data set).

Table 2.6 – Variable importance and coefficients of the top 20 variables for the sPLS model.

Gene	Variable Importance	Coefficient
CFAP61	100	-1.94
PCDHGA10	99.3	-0.83
PCDHGB5	97.5	-1.18
PCDHGB1	95.1	-1.02
CTNNBIP1	91.9	1.13
USP54	90.6	-0.86
PCDHGA8	83.5	-0.91
PCDHGB7	82.4	-0.80
MYOZ2	81.3	-0.61
PCDHGA11	78.5	-0.64
C12orf75	78.2	-0.82
PCDHGA7	77.8	-0.86
PCDHGA2	76.4	-0.92
HEXIM2	76.0	0.24
GRSF1	75.2	0.39
GCNT2	75.1	-1.07
FBP2	72.3	0.26
PLEKHO1	68.5	0.24
CRY2	68.1	-1.60
PABPC4	67.5	0.85

Discussion

In this study we compared the transcriptomes of skeletal muscle of healthy young, healthy older, and frail older subjects to better understand the skeletal muscle related part of the frail phenotype. A schematic overview of our findings is presented in figure 2.5. To our knowledge this is the first study investigating the effect of age on the muscle transcriptome to include frail and pre-frail older subjects. We observed clear and pronounced differences at baseline between young and older subjects. In our data frailty seems to present itself in the muscle transcriptome primarily as a more advanced stage of ageing (see also supplementary figures S2.3 and S2.4). This may, at least partly, be due to the higher average age of the frail group. There is, however, still significant overlap in age due to the high variation in age in both groups (79.8 ± 8.9, 74.1 ± 4.5 mean age and standard deviation for frail and healthy older subjects respectively, table 2.1 and 2.2).

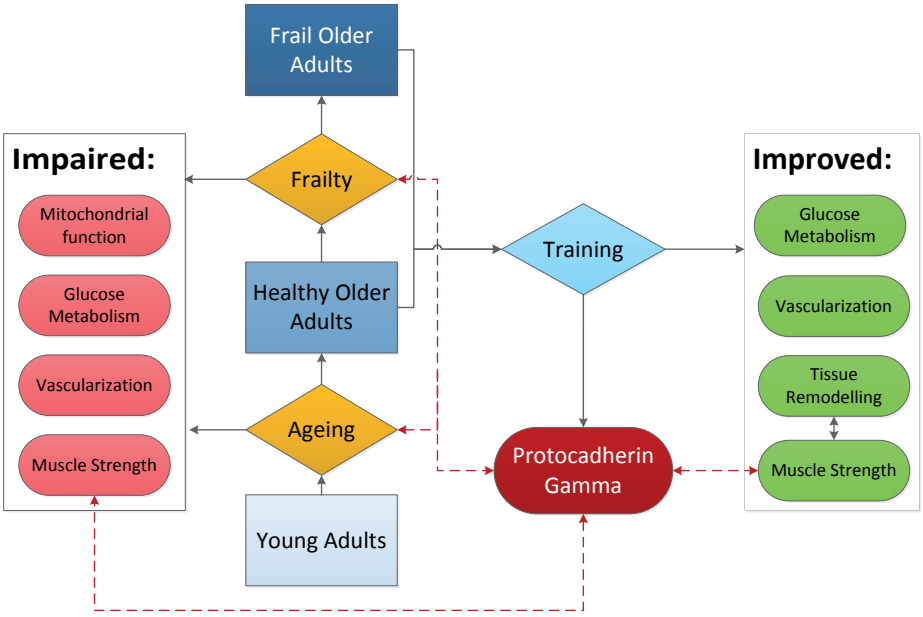

Figure 2.5 – Schematic summary of our findings.

Baseline differences between young and older subjects

There were significant differences in genes related to mitochondrial function and oxidative phosphorylation (supplementary figure S2.2). It is well known that mitochondrial function is impaired in older adults [5] which can be a responsive feature to muscle inactivity [28] and mitochondrial protein carbonylation [29]. In this case the average expression of mitochondrial genes is lowest in the frail older subjects at baseline. These expression differences may also represent a lower abundance of mitochondria [6].

There are two genes among the top differentially expressed genes between the three baseline groups that are as yet unknown, C20orf26 (CFAP61) and C12orf75. Both these genes have a higher expression levels in the older subjects and training appears to attenuate expression of these genes. Not much is known about the function of CFAP61 except that it is highly expressed in skeletal muscle tissue and may be related to calcium signalling and/or energy conversion [30]. C12orf75 may be related to cell proliferation and stem cell signalling [31].

In our data frail subjects showed significantly higher expression levels of METTL21C when compared to both the young and healthy older subjects, with a greater fold change difference between healthy and frail subjects than between the young and the older subjects (table 2.5). Training decreases the mean expression of METTL21C in both groups, but this does not reach significance using our significance cut-off. However, in the frail group it does reach a q-value of 0.08 after training, showing a fold change of -1.58. METTL21C encodes

for a protein-lysine methyltransferase belonging to a group of proteins that are involved in methylation of chaperone proteins, where METTL21C appears to methylate HSP70 and HSP90 [32] and has recently been found to be associated with skeletal muscle development [33]. *In vitro* inhibition of METTL21C expression in myoblasts showed impaired myotube differentiation and calcium signalling, suggesting that METTL21C plays an important role in the function of muscle cells and possibly also the overall quality of the muscle.

Effect of prolonged resistance–type exercise training

The majority of genes that significantly changed following prolonged resistance-type exercise training showed a shift in the expression levels towards levels observed in the younger group (figure 2.3). A previous study has shown a similar effect[14]. Indeed, Melov *et al.* state that training reverses the effect of ageing. While there is a shift towards younger expression levels, this does not necessarily mean that there is reversal of ageing. A more likely explanation is that the skeletal muscle in these older subjects have been 'detrained' due to more sedentary lifestyle when compared with healthy younger controls. Physical inactivity is a major contributor to age-related muscle loss and weakness and is one of the criteria of frailty [2]. In this way participation in prolonged resistance-type exercise training is likely to shift gene expression to younger levels. Furthermore, in our data the young subjects had higher muscle strength (table 2.1). Training leads to subtle but consistent changes in the muscle transcriptome [34]. Thus, a shift towards younger expression levels would be consistent with the increased strength after more prolonged resistance-type exercise training.

The genes that shift towards younger expression levels include genes related to the extracellular matrix, vascularisation, glucose metabolism and muscle contraction (supplementary figures S2.2, S2.5 and S2.6). The muscle biopsies were taken at least three days after the last training session. Thus, we are not observing acute effects of a single bout of resistance-type exercise, but rather longer term consistent changes in gene expression. Notably absent among the changes induced by prolonged resistance-type exercise training, however, are the primary differences observed when we compare young and older subjects: mitochondrial function. Possible explanations are that these changes are too subtle to pick up after 24 weeks of resistance-type exercise training or that prolonged resistance-type exercise training does not significantly affect these genes. Timing of the muscle biopsies relative to the last training session may also be a factor. It may be that expression of these mitochondrial genes only change acutely after resistance-type exercise rather than chronically.

Prolonged resistance-type exercise training showed fewer significantly affected genes in the frail group. Part of this can be explained by the differences in treatment. The healthy older subjects had training sessions three times per week whereas the frail older subjects received two sessions per week. The load of the training was also lower in for the frail subjects. However, it may also be that the frail are less capable of adapting to the additional stress of prolonged resistance-type exercise training. Fortunately, the frail subjects still showed a significant response to the training stimulus despite their less adaptive phenotype

[2]. Others have already reported that older adults in general have a decreased response to resistance-type exercise on a transcriptome level [35], and this may also play a role in the smaller response in the frail older subjects compared to the healthy older subjects.

Gene expression and muscle strength

Prolonged resistance-type exercise training led to strength increases in all individuals to the point that training increased strength levels in the frail older subjects close to the levels observed in the healthy older subjects at baseline (Figure 2.1). However, it did not necessarily lead to increases in lean body mass in all individuals (figure 2.2). This suggests that we primarily observed an increase in muscle quality, cross-bridge cycling efficiency, calcium handling and/or neuromuscular adaptation rather than an increase in muscle cross-sectional area. Our data provide evidence suggesting disturbances in axon guidance and muscle innervation in the older subjects.

We performed sPLS regression analysis to calculate leg strength based on expression levels of the genes that are robustly changed after prolonged exercise training in both groups in the baseline samples. Our rationale was that since these genes are changed in both groups after training, where the leg extension 1RM is significantly higher, that expression of these genes could also reflect muscle strength at baseline without training. We were able to build a reasonably accurate regression model to calculate leg extension 1RM at baseline based on gene expression (mean cross-validation R^2 of 0.73 and RMSE of 17.7, figure 2.4). Thus, expression of the genes robustly changed by exercise also seems to be indicative of muscle strength, not only after training but also prior to prolonged exercise training. This suggests that expression of these genes may be used as a biomarker to training status prior to study entry.

Several of the most important variables in our sPLS model for muscle strength belonged to the protocadherin gene cluster. Genes of the protocadherin gamma gene cluster were significantly different between frail older subjects, healthy older subjects and young subjects at baseline. Expression of these genes also went down after training in both groups. Older subjects had higher expression of this gene than young subjects and expression was highest in the frail older subjects. There are good indications that this gene cluster is relevant for neuromuscular performance. Many of the genes from this cluster are also significantly changed after training in both groups. Protocadherin gamma genes ranked very highly in the variable importance for our correlative model for leg extension 1RM (table 2.6).

Protocadherin gamma genes are primarily expressed in neural tissues such as the brain and the spinal cord and appears to be involved in axonal guidance [36]. Protocadherin proteins show homeophilic binding to other protocadherin proteins and in this way these proteins provide recognition sites for axonal binding. By expressing different protocadherin gamma genes from the gene cluster axons can be guided to different locations [37]. In knockout mice these genes appear to be indirectly related to muscle function: knock-out mice show severe muscle weakness and tremors, although this is attributed to loss of spinal motor neurons [38, 39]. It may also be that it is expressed at the muscle side of the synapse

to facilitate axon guidance towards muscle, and increased expression in this case is due to the denervation-reinnervation cycles seen in ageing muscle. Therefore, we hypothesise that as muscle loses innervation it increases expression of the protocadherin gamma cluster to facilitate axon binding from other nerves.

Protocadherin gamma is not the only significant group of genes related to neuromuscular function that we found in our data. There are several other genes differentially expressed between frail and healthy older subjects that are related to the innervation of muscle, including acetylcholine esterase (AChE) and kyphoscoliosis peptidase (KY). Both play important roles in the function of the neuromuscular junction [40, 41]. The top differentially expressed gene at baseline, unc-13 homolog C (UNC13C), is involved in neurotransmitter release [42, 43]. Both MYLK4 and IGFN1 are also among the top significantly different genes between the three groups at baseline. Little is known about MYLK4 and IGFN1, but both have been indirectly associated with neuromuscular function. MYLK4 has been shown to be significantly downregulated in AChE knockout mice, together with KY, suggesting that it is somehow involved in the signal transduction [44]. Like KY, IGFN1 has been associated with both muscle structure and neurological function [45, 46]. This gene also binds EEF1A2, which is the gene associated with the wasted (Wst) mouse phenotype [46, 47]. This phenotype shows significant immunological and neuromuscular defects [48]. IGFN1 has many splicing variants, which suggests that it plays a pleiotropic role in the muscle. Another indication of denervation is the increased expression of the perinatal myosin heavy chain isoform MYH8 in older subjects. Previous studies have found that expression of this gene is increased in tissue where the muscle fibres have lost innervation [49, 50].

Conclusions

Our data suggests a significant remaining plasticity of ageing skeletal muscle to adapt to regular resistance type exercise. Many age-related changes in skeletal muscle gene expression are partially reversed by prolonged resistance-type exercise training. Expression of the genes robustly changed following prolonged resistance-type exercise training in frail and healthy older subjects did not only reflect the effect of training itself, but also reflected muscle strength at baseline. Expression of the protocadherin gamma gene cluster is negatively correlated with muscle strength in our data and may be related to muscle denervation and re-innervation.

Clinical relevance

We have identified a gene cluster that may be related to denervation and re-innervation cycles in the muscle. Loss of motor neurons has been suggested to play an important role in age-related muscle weakness and sarcopenia, but is unfortunately not yet fully understood. Prolonged resistance-type exercise training was able to modulate the expression of protocadherin gamma. Hence, studying the expression of this gene may provide novel insights on whether or not denervation and re-innervation is modulated by interventions or lifestyle factors such as nutrition and physical activity. Furthermore, in this paper we show that steady-state gene expression provides information on the strength of the muscle itself. This suggests that steady-state gene expression could potentially be used as a tool to provide insight into muscle strength of a subject, but theoretically also other muscle health-related factors.

Limitations

While we have a large sample size for such a study, we also have a very heterogeneous study population. The ratio of male to female among both groups of older adults is not entirely equal. We have adjusted for this in our statistical analyses where possible. Another limitation is that the muscle biopsies from the frail older adults were obtained from a study performed at Wageningen University, whereas the muscle biopsies from the healthy older adults and the young adults were obtained from studies performed at Maastricht University. Protocols for muscle biopsy collection and preparation in Wageningen are based on those from Maastricht and thus are very similar, but there may still be some bias that we cannot account for statistically. The microarray analyses were performed within the same lab at the same time by the same technician, which means that batch effects should be minimal. The protocols for the prolonged resistance-type exercise training were slightly different for the frail older adults and the healthy older adults. The primary difference being that the healthy older adults trained three times per week and the frail older adults trained twice per week. As a consequence, the training stimulus for the frail older adults was somewhat lower in these individuals and this could partially explain the decreased response among the frail older subjects. This difference in treatment also prevented us to compare the training responses in both groups directly.

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Conflict of Interest

JA Timmons is a Founding Director of XR Genomics, a Personalised Health and Fitness company. John van Duynhoven is employed by a company that manufactures and markets food products. The other authors have no competing interests to declare.

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Chapter 3 - The muscle metabolome differs between healthy and frail subjects of older age

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Abstract

Populations around the world are aging rapidly and concomitant loss of physiological functions negatively affects quality of life at older age. A major contributor to the frailty syndrome of ageing is loss of skeletal muscle. In this study we assessed the skeletal muscle biopsy metabolome of healthy young, healthy older and frail older subjects to determine the effect of age and frailty on the metabolic signature of skeletal muscle tissue. Moreover, effects of prolonged whole-body resistance type exercise training on the muscle biopsy metabolome of older subjects were examined. The baseline metabolome was measured in muscle biopsies collected from 30 young, 66 healthy older subjects and 43 frail older subjects. Follow-up samples from frail older (24 samples) and healthy older subjects (38 samples) were collected after 6 months of prolonged resistance-type exercise training. Young subjects were included in order to have a reference for expected shifts of the older towards a younger metabolic phenotype. Primary differences in skeletal muscle metabolite levels between young and healthy older subjects were related to mitochondrial function, fiber type, and tissue turnover. Similar differences were observed when comparing frail with healthy older subjects. Prolonged resistance-type exercise training revealed a correlative adaptive response of amino acids, especially branched chain amino acids, and genes related to tissue remodeling. The impact of exercise on branched chain amino acids derived acylcarnitines in older subjects points to a downward shift in branched chain amino acid catabolism upon training. We observed modest correlations between muscle metabolite and plasma levels. This prohibits the use of the latter as read-outs of muscle metabolism and stresses the need for direct assessment in muscle tissue biopsies.

Introduction

Populations around the world are aging rapidly and concomitant loss of physiological functions compromises independence at older age. It has been estimated that people older than 60 y will make up 22% of the world population and people older than 80 y will account for 4.4% of the world population in 2050 [1]. A major contributor to the frailty syndrome of ageing is skeletal muscle loss, which can lead to increased disability in the older population. In most people muscle mass and strength start to decline around the age of 35 y with more progressive muscle loss observed after the age of 65 y [2-6].

From a recent study [7] we learned that prolonged resistance-type exercise training partially shifts the skeletal muscle transcriptome of older subjects toward an expression pattern observed in muscle tissue of young subjects, with changes in gene expression related to vascularisation, tissue remodelling and glucose metabolism. We hypothesize a similar shift towards the young phenotype in the muscle metabolome after resistance-type exercise training in older subjects. The transcriptome analysis also revealed substantial differences between healthy young men, healthy older subjects and frail older subjects before any intervention was undertaken. Particularly genes related to mitochondrial function were downregulated in older subjects compared to young.

Although the effects at gene expression level are expected to be reflected in metabolic regulation, our insights are limited to only few studies, mostly in animals. Recent work showed that ageing affects glucose and fatty acid metabolism in muscle of mice [8]. A study on aged rats suggests a muscle group-specific perturbation of lipid and glucose metabolism consistent with mitochondrial dysfunction [9]. A recent study in humans showed that lipid content and oxidative activity in skeletal muscle are related to muscle fiber type in ageing and metabolic syndrome [10]. Another human study showed that upon training mitochondrial function and intermediary metabolism were reprogrammed in insulin-insensitive obese subjects [11]. Within these human studies the deployed metabolic profiling platforms had limited coverage and focused on specific sub-metabolomes.

A major bottleneck in achieving extended coverage was the limited amount of muscle tissue material that can be obtained from human studies. The recent development of a comprehensive targeted metabolic profiling platform [12] optimized and validated for small muscle biopsies paved the way to understand the observed phenotypical differences at a more comprehensive level. Here we establish the effect of aging and frailty on the skeletal muscle metabolome. We further examine the impact of prolonged resistance-type exercise training on the metabolome of frail and healthy older subjects. We included a population of young subjects in our study in order to have a reference for expected shifts of the older towards a younger metabolic phenotype.

Materials and Methods

Experimental design

Muscle biopsies (*Vastus lateralis*) and circulating metabolites samples were collected from pre-frail, frail older [13, 14], healthy older [15, 16] and young subjects [17]. Medical history of all subjects was evaluated by medical questionnaires which were analyzed by a physician. Subjects who were unable to participate in the training due to pain, were excluded prior to starting the intervention. Included subjects who showed severe discomfort during the training sessions were excluded from further participation in the study. Baseline metabolite levels were measured in skeletal muscle tissue of 30 young, 66 healthy and 43 frail older subjects (Table 3.1). We also measured metabolites in plasma and serum for 50 young, 76 healthy and 62 frail older subjects (Supplementary Table S3.1). The transcriptome of our muscle biopsy set was measured in an earlier study[7].

Table 3.1. Characteristics of subjects of which skeletal muscle tissue biopsies were studied

	Young	Healthy older	Frail older
N (male / female)	30/0	47/19	25/18
Age (years)	21.7 ± 2.5	71.7 ± 5.2	77.5 ± 8.0
Height (m)	1.83 ± 0.06	1.72 ± 0.08	1.67 ± 0.09
Weight (kg)	76.7 ± 11.8	75.9 ± 13.3	77.5 ± 11.1
BMI[a] (kg / m^2)	22.6 ± 2.7	25.5 ± 3.0	27.5 ± 3.7
Body Fat (%)	14.9 ± 4.9	24.5 ± 5.6	32.1 ± 8.8

Data was presented as mean ±SD. a: body mass index.

Samples from healthy young male subjects were derived from several studies performed within our group, in which exactly the same technique and processing was used for sample collection. We used young male subjects as a reference group and samples were only taken at baseline. Samples from the frail and healthy older subjects were obtained from two study centers. More details of the studies can be found in the respective papers [13, 16].

In addition, muscle biopsies were obtained after 6 months of resistance-type exercise training for 38 healthy and 24 frail older subjects. All muscle biopsies and circulating metabolites samples were obtained in the morning, in an overnight fasted state, after standardized meal the evening before, and no strenuous physical activity for 3 days prior to muscle biopsy collection.

Fried criteria [18] were used to assess the frailty in older subjects, in which frailty is viewed in terms of the unintentional weight loss, weakness, self-reported exhaustion, slow walking speed, and low physical activity. Based on the above mentioned criteria, the healthy older subjects were not considered frail or pre-frail at the start of the intervention study [19]. In our study population, all older subjects regardless of their health status (frail or healthy) improved in muscle performance following 6 months resistance-type exercise training

(Supplementary Figure S3.1 Table S3.2) as illustrated by significantly increased leg extension and leg press strength after training (*P*-value < 0.01) [7, 13, 19]. Both healthy older and frail older subjects followed similar progressive full-body resistance type exercise training. In brief, the training consisted of a 5 minute warm-up on a cycle ergometer, followed by 4 sets on the leg-press and leg-extension machines. In addition, 3 sets on chest press, lat pulldown, ped-dec and vertical row machines were performed (Technogym, Rotterdam, The Netherlands). However, the healthy older subjects trained 3 times per week and frail subjects trained 2 times per week. Moreover, subjects received a protein or control supplement during the study. Healthy older subjects took 15 gram milk protein or control drink at breakfast and frail older subjects took a similar 15 gram drink (milk protein or control) at breakfast and lunch every day throughout the entire 6 months intervention. Full details can be found in the earlier papers on the phenotypical impact of training on our study population [13, 19].All studies were approved by The Medical Ethics Committee of either Wageningen University or Maastricht University and comply with the Declaration of Helsinki.

Metabolomics analysis of circulating metabolites

Amino acids and biogenic amines were derivatized (Acc-Tag) in 5 µL aliquots of plasma. Samples were analyzed using an ACQUITY UPLC system with autosampler (Waters, Etten-Leur, The Netherlands) coupled with a Xevo Tandem quadrupole mass spectrometer (Waters) operated using QuanLynx data acquisition software (version 4.1; Waters). An Accq-Tag Ultra column (Waters) was used. The Xevo TQ was used in the positive-ion electrospray mode and all analytes were monitored in Multiple Reaction Monitoring (MRM) using nominal mass resolution. Acquired data were evaluated using TargetLynx software (Waters), by integration of assigned MRM peaks and normalization using proper internal standards[20].

Acylcarnitines, trimethylamine-N-oxide, choline, betaine, deoxycarnitine and carnitine were analyzed in 5 µL plasma, spiked with an internal standard, using a UPLC-MS/MS. Also here an Accq-Tag Ultra column was used. The Xevo TQ was used in the positive-ion electrospray mode and all analytes were monitored in Multiple Reaction Monitoring (MRM) using nominal mass resolution. In-house developed algorithms [21] were applied using the pooled QC samples to compensate for shifts in the sensitivity of the mass spectrometer over the batch.

Organic acids were measured by GC-MS using 50 µL of plasma sample prepared using a two-step derivatization procedure with subsequent oximation using methoxyamine hydrochloride (MeOX) and silylation using N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA. Samples were measured on an Agilent GC (7890A) coupled to Agilent Quadrupole-MS with EI source (Agilent MSD 5975C). Separation was performed using a HP-5MS column (30 m x 0.25 m x 0.25 µm; Agilent). The raw data were pre-processed using Agilent MassHunter Quantitative Analysis software for GC-MS (Agilent, Version B.04.00), and quantitation of metabolite response was calculated as the peak area ratios of the target analyte to the respective internal standard. In-house developed algorithms were applied using the pooled

QC samples to compensate for shifts in the sensitivity of the mass spectrometer over the batch. Serum metabolite concentrations determined by NMR were measured as described by Mihaleva *et al.* [22]. In short, serum samples were ultrafiltrated and automated quantum mechanical line shape fitting of ^{1}H NMR spectra was performed using PERCH.

In tissue metabolome analysis

Metabolites were extracted from 10 mg of wet muscle tissue. This tissue was further lyophilized and weighted to determine the dry tissue mass. After pulverizing the tissue, metabolites were extracted using methanol/chloroform/water (MCW). The extraction method used in this study has extensively been described and characterized elsewhere [12].

Amines, acylcarnitines and oxylipins were measured using the platforms also used for measurement of these metabolites in plasma. The validation of these methods for human tissue biopsies is described in detail elsewhere [21]. To compensate for shifts in the sensitivity of the mass spectrometer over multiple batches of measurements, in-house developed algorithms were applied [21]. The metabolite response was determined by the peak area ratio of the target analyte to the appropriate internal standard. These response ratios were used in the subsequent data analysis. ATP, ADP, creatine and phosphocreatine were determined spectrophotometrically using established enzymatic assays. For the first 3 metabolites, commercially available fluorimetric assay kits were used (BioVision cat.# K354-100, K355-100 and K635-100) following the manufacturer's instructions. Phosphocreatine was measured according to a colorimetric assay kit protocol described by Szas *et al*.[23]. In total 96 metabolites including amine, acylcarnitines, organic acids, oxylipins and a number of nucleotides were measured.

Statistical analysis

Statistical analysis was performed on log-transformed data. We used analysis of variance (ANOVA) for between group comparisons at baseline. *P*-value<0.05 was considered significant. We used linear mixed models for assessment of the training effect. Our model included exercise training, subject, sex, protein supplementation and within subject correlation. Analyses of the training effect was performed separately for frail and healthy older subjects due to differences in training frequency. To summarize acylcarnitines into one single metabolite (eigen metabolite), the Non-linear Iterative partial least squares (NIPALS) algorithm [24] of the mixOmics R library was used to calculate the Singular Value Decomposition (SVD) of acylcarnitines. The mixOmics R library was used to perform multilevel sparse partial least squares (sPLS) [25, 26]for integration of metabolomics and transcriptomics. A canonical correlation cut-off of 0.80 was used for building the network. FactoMineR was used to perform principal component analysis (PCA) [27]. All analyses were done using R (version 3.02).

ASCA (ANOVA Simultaneous Component Analysis) was performed to determine global differences on metabolites. ASCA is a multivariate method that partitions variation in the data and enables to interpret these partitions by simultaneous component analysis [28]. Analysis

was performed separately for the frail and healthy older subjects, using delta values of each metabolite for each individual (value after training - value before training) with supplement, sex and their interaction in the model as factors. Analysis was done under Matlab (version R2012a).

Results

Baseline comparisons between healthy older, frail older and young subjects

Comparison of the circulating metabolites profiles of young and healthy older subjects revealed a range of metabolites that differed significantly between both groups. Analysis of variance (ANOVA) showed that amino acids and acylcarnitines were responsible for the main differences in circulating metabolites between older and young subjects. These differences were in line with previous observations of age-related effects on the circulating metabolome [8, 29, 30].

Figure 3.1 shows a principal component analysis (PCA) of the biogenic amine profiles of the muscle biopsies obtained from young, healthy older and frail older subjects, before and after exercise. We observed clear age-related differences in the biogenic amine profiles of skeletal muscle, as well as a difference between healthy and frail older subjects. The PCA plot also revealed an effect of prolonged resistance-type exercise training on amine concentrations in muscle tissue. PCA revealed a similar effect for organic acids in muscle (Supplementary Figure S3.2).

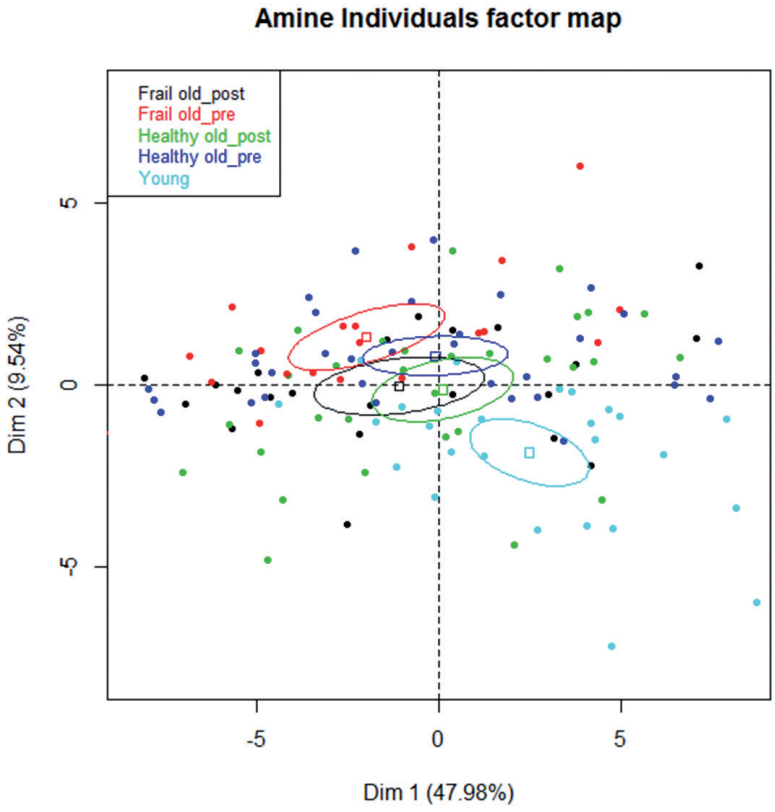

Figure 3.1. Principal component analysis (PCA) plot of biogenic amines detected in muscle biopsies. To visualize whether groups are significantly different from each other, confidence ellipses (95% Confidence Interval) were drawn around them.

ANOVA yielded a series of skeletal muscle metabolites that significantly differ between healthy older subjects and young subjects. Many of these metabolites are amino acids and organic acids (Table 3.2). The outcome of ANOVA modeling of muscle metabolites in healthy vs. frail older subjects is presented in Table 3.3.

Table 3.2. Muscle metabolites that are significantly different between healthy older and young men.
a: Out of a comprehensive muscle biopsy metabolic profile those metabolites are presented that are significantly (*P*-value =<0.05) different between young and older subjects according to univariate ANOVA models. b: Fold Change

Metabolite	*P*-value	FC[b] (Older/Young)
TCA Cycle		
Succinic acid	0.02	0.76
2-ketoglutaric acid	0.03	0.76
Fumaric acid	0.04	0.82
Lactic acid	0.05	0.69
Energy		
ATP	<0.01	0.75
ADP	0.01	0.88
Branched chain amino acids		
Valine	<0.01	0.81
Leucine	<0.01	0.81
Isoleucine	0.03	0.84
Acylcarnitines		
Acetylcarnitine (C2)	<0.01	0.49
Malonylcarnitine (C3-DC)	<0.01	0.46
Intracellular buffering		
Carnosine	<0.01	0.7
Arginine, Proline Pathway		
Ornithine	<0.01	1.55
Arginine	<0.01	1.34
4-hydroxy-proline	0.01	0.69
Proline	0.02	0.84
Glycylglycine	0.05	0.87
Methionine	<0.01	0.8
Other amino acids		
Lysine	<0.01	1.44
Aspartic acid	<0.01	1.45
Oxylipins		
LA (LOX)		
9-HODE	<0.01	1.4
13-HODE	0.01	1.37
13-KODE	0.03	1.3
LA(CYP450)		
9,10-EpOME	0.04	1.39
ALA (LOX)		
9-HOTrE	0.01	1.71
DGLA (LOX)		
15S-HETrE	0.02	0.83
8-HETrE	0.03	0.85
Aminobutyric acids		
βamino isobutyric acid	<0.01	1.61
βaminobutyric acid	0.01	0.82

Table 3.3. Muscle metabolites that are significantly different between frail and healthy older subjects. a: Out of a comprehensive muscle biopsy metabolic profile those metabolites are presented that are significantly (*P*-value =<0.05) different between frail and healthy older subjects according to a univariate ANOVA models that accounted for Group effects (Frail vs. Healthy), Sex effect, and Group and Sex interaction; Significance of Group (Frail vs. Healthy older subjects) and Group & Sex interactions has been indicated; b:fold change; c: not significant (NS)

Metabolite	Group	Sex	Interaction	FC[b](Frail/Healthy)
TCA Cycle				
Citric acid	<0.01	NS [c]	NS	0.54
Acylcarnitines				
Isovalerylcarnitine (C5)	<0.01	NS	NS	0.42
Octenoylcarnitine (C8)	0.03	0.03	NS	0.77
Malonylcarnitine(C3-DC)	0.02	NS	NS	0.77
Carnitine (C0)	0.01	NS	NS	0.75
Intracellular buffering				
Carnosine	0.01	NS	NS	0.8
Oxylipins				
LA (CYP450)				
12,13DiHOME	0.04	NS	0.03	1.18
DGLA (LOX)				
8HETrE	0.03	NS	NS	0.81
15SHETrE	<0.01	NS	NS	0.77
Polyamine metabolism				
Spermidine	0.01	0.02	NS	1.24
Spermine	0.04	NS	NS	0.9
Other amino acids				
Histidine	<0.01	NS	NS	0.79
Asparagine	0.01	NS	NS	0.81
Taurine	0.01	NS	NS	0.79
Serine	0.01	NS	NS	0.86
Glycine	0.02	NS	NS	0.81
oacetylserine	0.02	NS	NS	0.9
Homoserine	0.02	NS	NS	0.85
Tyrosine	0.02	NS	NS	0.83
Tryptophan	0.02	0.04	NS	0.83
Methionine	0.02	NS	NS	0.83
Glutamine	0.03	NS	NS	0.82
Pyroglutamic acid	0.03	NS	NS	0.83
Glutamic acid	0.04	NS	NS	0.82
Glycylglycine	<0.01	NS	NS	0.77
Aminobutyric acids				
βamino isobutyric acid	0.05	NS	NS	0.79

Effect of prolonged resistance–type exercise training in frail and healthy older subjects

In the PCA plot in Figure 3.1 we can observe that prolonged resistance-type exercise training has an effect on the biogenic amine profile of skeletal muscle tissue of frail and healthy older subjects. The trends in the PCA plot suggest that upon training both healthy and frail older subjects shift towards a younger phenotype (see also the plot for the average value of isoleucine in Supplementary Figure S3.3a). The variation in this PCA plot may not only be due to phenotype (young, healthy and frail old) and exercise, but also to sex and protein supplementation. Hence we performed ANOVA Simultaneous Component Analysis (ASCA) to account for these different sources of biological variation. We observed that prolonged resistance-type exercise training had a weak effect on muscle tissue metabolite levels. No significant interaction of protein supplementation with training in both frail and healthy older subjects could be observed. Interaction of sex with training was not significant in healthy older subjects, but was significant in frail older subjects (*P*-value=0.03).

Next we used linear mixed models on univariate metabolite levels to account for phenotype, exercise, protein supplementation and sex as sources of biological variation. We observed that prolonged resistance-type exercise training affected muscle levels of acylcarnitines in both the healthy older and frail older subjects (Table 3.4 and 3.5, respectively). These effects pertain to propionyl (C3), methylmalonyl (C4-DC), and isovaleryl (C5) acylcarnitines as products of the oxidation of amino acids, branched chain amino acids (BCAA), and C6-C20 acylcarnitines as products of fatty acid oxidation [31]. For several acylcarnitines the exercise effects were nearly significant (*P*-value >0.05), hence we used the singular value decomposition to summarize the levels of fatty acid derived and amino acid derived acylcarnitines (denoted as FAAC and AAAC, respectively in Figure 3.4 and 3.5). Subsequently, we also performed linear mixed models to determine the effect of training on the fatty acid derived and amino acid derived acylcarnitines. The amino acid derived acylcarnitines levels were significantly decreased after training for both healthy and frail older subjects (see example of the effects for propionylcarnitine in Supplementary Figure S3.3, b).

Table 3.4. Training effect on muscle metabolites in healthy older subjects [a] a: Metabolites are presented that significantly (*P*-value =<0.05) differ pre- and Post-training in healthy older subjects according to univariate linear mixed models.We note that we constructed also linear mixed models that account for other factors and their interactions, but these were not found to be significant. b: fold change, c,d: Single Value Decomposition (SVD) were calculated for amino and fatty acid acylcarnitines, denoted as AAAC and FAAC, respectively.

Metabolite	Training	FC(post/pre training)[b]
Pipecolic acid	0.002	1.64
Isovalerylcarnitine (C5)	0.005	0.56
Linoleylcarnitine (C18:2)	0.01	0.61
Oleylcarnitine (C18:1)	0.01	0.7
Propionylcarnitine (C3)	0.01	0.73
Palmitoylcarnitine (C16)	0.02	0.75
11.12.EpETrE	0.03	1.26
Tetradecenoylcarnitine (C14:1	0.03	0.47
AAAC[c]	0.02	0.77
FAAC[d]	NS	0.96

Table 3.5. Training effect on muscle metabolites in frail older subjects a: Metabolites are presented that significantly (*P*-value =<0.05) differ pre- and post-training in frail older subjects according to univariate linear mixed models that account for Supplement, Sex and (Training and Supplement) Interaction. We note that we constructed also linear mixed models that account for other interactions, but these were not found to be significant. b: fold change, c: Not Significant, d & e: Single Value Decomposition (SVD) were calculated for amino acid and fatty acid acylcarnitines (AAAC and FAAC, respectively).

Metabolite	Training	Supplement	Sex	Interaction	FC (post/pre training)[b]
Propionylcarnitine (C3)	<0.01	NS[c]	NS	NS	0.75
Glucose	<0.01	NS	<0.01	NS	1.35
Lactic acid	0.01	NS	<0.01	NS	1.55
Tetradecenoylcarnitine (C14:1)	0.03	NS	NS	NS	2.00
Methionine	0.04	<0.01	NS	NS	1.22
Tryptophan	0.04	NS	NS	NS	1.21
β Alanine	0.05	NS	NS	0.01	0.92
Isoleucine	0.05	<0.01	0.02	NS	1.21
Myristoylcarnitine (C14)	0.05	NS	NS	NS	1.73
AAAC[d]	0.01	NS	NS	NS	0.76
FAAC[e]	NS	NS	NS	NS	1.95

We used multilevel sPLS to integrate muscle changes in the transcriptome and metabolome after training. Here the goal was to investigate the interaction of two matched data sets and the selection of subsets of either positively or negatively correlated variables across all subjects. This multivariate approach highlighted the training effects within subjects separately from the biological variation between subjects. We applied canonical mode, which highlights the strongest correlations between the two data sets. Subsequently, we selected significantly changed genes based on training (FDR<0.05) in both frail and healthy older subjects. We observed that amino acids, particularly branched chain amino acids, correlate with genes related to connective tissue/ extracellular matrix such as collagen, laminin and SPARC (Figure 3.2).

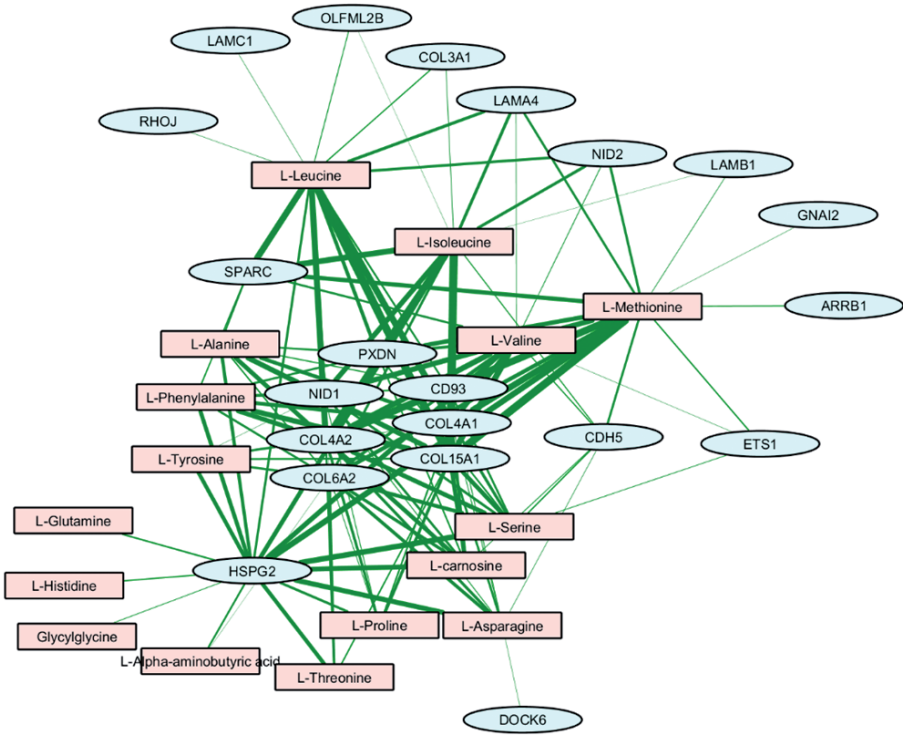

Figure 3.2. Correlation network of muscle metabolites and genes.
Only significantly changed genes were selected (FDR<0.05). Metabolite canonical correlation cutoff <= 0.80. Circle: gene, rectangle: metabolite. Green: positive correlation.

Correlation between muscle and plasma metabolites levels

Within this study we simultaneously collected fasting plasma and muscle biopsies. We investigated to what extent baseline plasma metabolite levels reflect muscle metabolite levels. We first constructed separate correlation heatmaps for muscle and plasma metabolites within each compartment. In Figure 3.3a-b one can observe that metabolites that are in the same group of metabolites (e.g. amino acids, acylcarnitines) are correlated to each other within both plasma and muscle. However, the correlation heatmap of muscle and plasma metabolites (Figure 3.3c) showed only minor to moderate correlations between muscle and plasma metabolites (Pearson correlation between 0.3 and 0.5). The correlation networks (Figure 3.3d) show that strongest correlations pertain to 3-hydroxybutyric acid, 4-hydroxyproline, proline, branched chain amino acids and several acylcarnitines. Correlation of serum and muscle metabolites are presented in Supplementary Figure S3.4. Results are generally in line with plasma-muscle metabolite correlations.

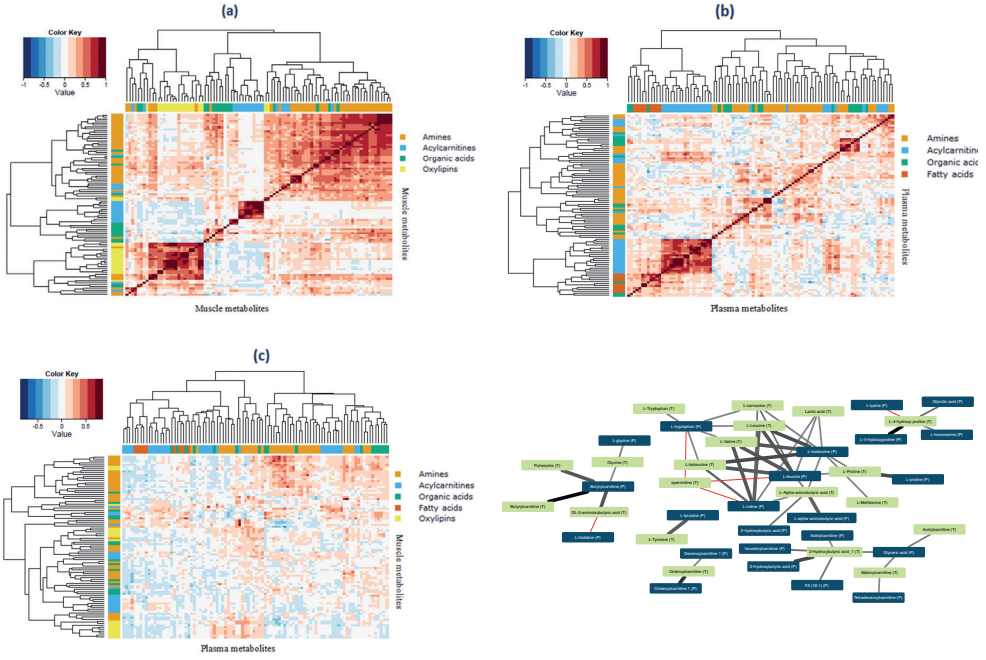

Figure 3.3 - Correlation heatmap of muscle to muscle metabolites (a), plasma to plasma metabolites (b), muscle to plasma metabolites (c). Pearson correlation were used. (d) Correlation network of the most strongly correlated muscle and plasma metabolites (Pearson correlation). Red and blue indicate positive and negative correlations, respectively. Thick lines: correlation ~0.5, thin lines: 0.3 <correlation <0.5. Pink nodes: muscle tissue (T) metabolites and yellow nodes: plasma (P) metabolites.

Discussion

Comprehensive metabolic profiling of muscle biopsies: age and resistance–type exercise training effects in a heterogeneous study population

Comprehensive metabolic profiling of muscle biopsies revealed baseline differences between the muscle biopsy metabolomes of healthy young, healthy older and frail older subjects. Furthermore, distinct effects of prolonged resistance-type exercise training in the latter two groups could be observed. Critical was the deployment of five robust profiling platforms using a single and thoroughly validated muscle-biopsy extraction procedure [12]. The analytical variation in these profiles, was around 20-30% which was smaller than the biological variation in the muscle biopsies. In order to separate the sources of biological variation in our heterogeneous study population we relied on univariate linear mixed models. The metabolic effects of sex and supplementation however turned out to be relatively minor compared to phenotype and response to prolonged resistance-type exercise training. A multivariate approach (ASCA) did not show any significant effects of training, probably because not all sources of biological variation could adequately be accounted for [32]. In the following discussion we limit ourselves to univariate approach since it more strictly accounts for heterogeneity of our study population.

Baseline comparisons

TCA cycle metabolites (succinic acid, fumaric acid, 2-ketoglutaric acid) were lower in the healthy older subjects compared to young subjects. These differences were accompanied by lower levels of ATP, ADP, branched chain amino acids and acylcarnitines in the healthy older subjects. This indicates impaired mitochondrial function or a lower number of mitochondria in the muscle of the older subjects. It may also be a reflection of the lower habitual physical activity of the older subjects. These observations are in line with data on the transcriptomics profile of these subjects, which show that genes related to mitochondrial function and oxidative phosphorylation have decreased expression in the older subjects compared to young subjects, with the lowest expression occurring in the frail older subjects [7]. This is also in agreement with other studies [8, 33-37], where the mitochondrial electron-transport chain is also among the significantly different pathways in muscle metabolism when comparing older and young subjects.

Worth mentioning are the lower levels of 4-hydroxyproline and proline in the healthy older subjects compared to the young subjects. Both of these amino acids have been associated with collagen turnover [38, 39]. Two precursors of proline, ornithine and arginine, have higher muscle levels in the older subjects. Higher levels of ornithine and arginine, together with lower levels of 4-hydroxyproline and proline, may be due to dysfunction of the mitochondrial ornithine aminotransferase [40], leading to accumulation of arginine and ornithine. This is in line with slightly lower expression of genes related to tissue remodeling, such as collagen in the older subjects compared to the young subjects [7].

In healthy older subjects we observed higher muscle levels of β-isoamino butyric acid (BAIBA) than in young subjects. Hypothetically, this metabolite is produced upon exercise by expression of PGC-1α and has been proposed as a myokine stimulating browning of white adipose tissue and hepatic β-oxidation [41]. In our study population we observe gene transcription downregulation of PGC-1α target genes in healthy older subjects with respect to young and in frail older subjects with respect to healthy older subjects [7]. At posttranslational level activity of PGC-1α is modulated by NAD^+ dependent deacetylation by SIRT1 (Figure 3.4-b) [42]. Since NAD^+ levels are decreasing with age [43], we expect also here a downregulation of PGC-1α [44]. The expected decrease of β-isoamino butyric acid (BAIBA) levels is however only observed when comparing frail to healthy older subjects, whereas we observe a significant increase in BAIBA in healthy older subjects compared to young subjects. Thus, the observed changes in baseline levels of BAIBA levels in muscle in our study population do not appear to be consistent with age-related down regulation of PGC-1α. Hence we cannot confirm the recently stated hypothesis that BAIBA acts as a PGC-1α induced myokine. An explanation could be that in our study differences in PGC-1α expression are caused by age and frailty dependent processes, whereas previously described PGC-1α -mediated effects on BAIBA were caused by acute exercise.

Two polyamines, spermine and spermidine, were found to be significantly different in the frail compared to the healthy older subjects. Polyamines are involved in tissue regeneration and cell proliferation and differences are associated with both exercise and muscle pathology [45-47]. The genes directly involved in the polyamine pathway are however not differentially expressed between frail and healthy older subjects. Hence the observed differences in polyamine levels between the frail and healthy older subjects are more likely to be attributed to effects at the level of enzyme activity or metabolite transport. Previous studies have shown that perturbations in polyamine metabolism are associated with neuromuscular disorders [48, 49]. On the transcriptome level we indeed found indications of neuromuscular perturbations in the frail older subjects [7]. In addition, increased spermine levels were recently linked to skeletal muscle atrophy [50]. However, our data suggests that at baseline this process is in the opposite direction when comparing frail and healthy older subjects, even though frail older subjects generally have less skeletal muscle and are likely to exhibit more extensive muscle atrophy. Ost *et al.* recently reported that spermidine is increased in the skeletal muscle of mice overexpressing uncoupling protein 1. The authors proposed that this might be an adaptive response to cope with the additional oxidative stress [51].

The level of carnosine is decreased in healthy older subjects relative to young subjects, and in frail relative to healthy older subjects. Carnosine is an abundant metabolite in muscle where it plays an important role in intracellular pH buffering [52]. Carnosine has also been associated with chelation of metal ions and antioxidant activity [53]. Carnosine levels are higher in type II muscle fibers compared to type I. A likely explanation for the

significantly lower levels of carnosine in healthy and frail older subjects is therefore the decrease of muscle fiber II/I ratio with respectively age and lack of exercise[54].

Several oxylipins derived from linoleic acid (LA) and α-linoleic acid (ALA) occur at higher levels in the muscle of the healthy older subjects compared to young subjects. On the other hand, metabolites derived from the Δ-6 desaturase product dihomo-γ-linoleic acid (DGLA) are reduced in healthy older subjects. We postulate that due to reduced Δ-6 desaturase activity linoleic acid and α-linoleic acid accumulate in the muscle of the healthy older subjects, whereas downstream Δ-6 desaturase product dihomo-γ-linoleic acid products are depleted [55].

Effect of prolonged resistance–type exercise training

We compared the effect of prolonged resistance-type exercise training in both healthy and frail older subjects with all metabolites using multilevel sPLS. There was a profound correlation between the adaptive response to training between the transcriptome and amino acids in the muscle metabolome (canonical correlations between 0.7 and 0.8). There were particularly high correlations between expression changes of extracellular matrix genes and amino acids. Although it is unlikely that there is a direct link between expression of these genes and levels of these metabolites, it does imply that these changes in amino acid levels are part of the adaptive response to resistance-type exercise training.

At the metabolite level, the most striking effects of resistance-type exercise training in frail and healthy older subjects were observed for the C3 (propionyl) and C5 (isovaleryl) muscle acylcarnitines derived from branched chain amino acids. After training, the amino acid derived acylcarnitines showed a significant decrease both in the healthy and frail older subjects, accompanied by an increase of branched chain amino acids. A likely explanation is that the flux-determining mitochondrial branched chain α-keto acid hydrogenase (BCKDH) complex [56] has a compromised response to prolonged resistance-type exercise training. As is schematically depicted in Figure 3.4-a, the BCKDH complex can respond to exercise via different mechanisms. PGC-1 is a known activator of BCKDH, but training did not have an effect on its gene expression in our study. Exercise is known to increase NAD^+ levels [44] and could thus activate PGC-1α in a post-translational manner via SIRT1. Apparently also this mechanism is not activated by training in the older subjects. These effects are specific for branched chain amino acids oxidation and no significant effects on fatty acid derived acylcarnitines were found. A decrease in branched chain amino acids oxidation may stimulate mTOR related pathways activation and protein synthesis [57, 58], which is beneficial for older subjects. As this mechanism occurs at enzymatic level, the available metabolomics and transcriptomics data can however not confirm this hypothesis and in future studies proteomics would be called for.

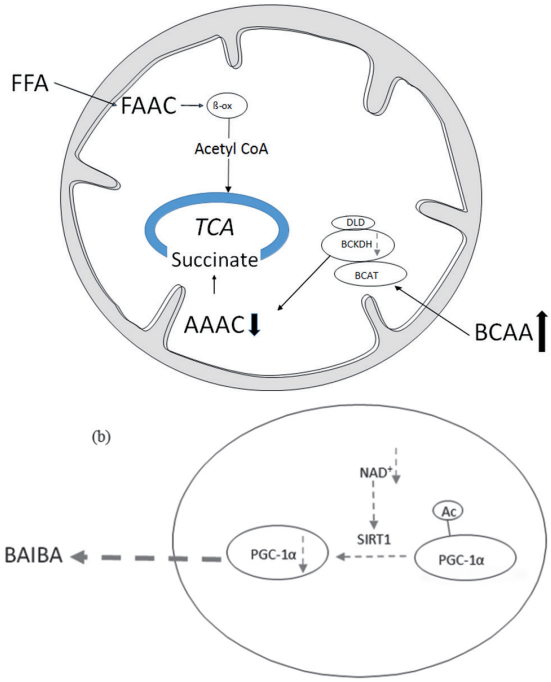

Figure 3.4 - (a) Schematic representation of mitochondrial oxidation of free fatty acids (FFA) and branched chain amino acids (BCAA). Arrows indicate effect of prolonged resistance-type exercise training on older subjects (healthy and frail) on BCAA (increase) and acylcarnitines (decrease) as well as the proposed (dashed-arrows) downregulation of the branched chain α-keto acid hydrogenase (BCKDH) complex. (b) Schematic representation of age-related NAD+ dependent acetylation of PGC1α. Dashed arrows indicate NAD+ and SIRT1 dependent downregulation of PGC-1α and β-isoamino butyric acid (BAIBA).

Correlation of plasma and muscle metabolome

The weak correlations between plasma and muscle metabolite levels indicate that plasma levels only partially reflect muscle metabolism, even though muscle is one of the largest metabolically active tissues in the human body. This seems to suggest that these metabolites are also produced by other metabolic compartments. In a recent study, lack of correlation between acylcarnitine levels in plasma and tissues in mice was attributed to differences in turnover in plasma and muscle compartments, and contribution of other compartments than muscle to plasma acylcarnitine levels [59]. The same rationale very likely also applies to amino acids involved in collagen metabolism (proline, 4-hydroxyproline), which can also be formed in bone. Adipose tissue is also a metabolically active compartment for branched chain amino acids besides muscle [60], and this will likely weaken plasma-muscle level correlations. As a consequence, correlations between plasma metabolites and muscle metabolites are too modest to support their use as direct read-outs of muscle metabolism [61, 62].

Conclusion

The major differences in muscle metabolome of healthy older and young subjects relate to mitochondrial function, fiber-type composition, and tissue turnover. Similar differences were observed when comparing frail older subjects with healthy older subjects. Prolonged resistance-type exercise training showed a correlative adaptive response of amino acids and genes responsible for tissue remodeling. The effect of exercise on amino acid derived acylcarnitines in healthy and frail older subjects points towards decreased branched chain amino acids catabolism likely due to attenuated activation of the flux-determining mitochondrial branched chain α-keto acid hydrogenase complex in older subjects. Only modest correlations between muscle metabolite and plasma levels were found, which prohibits the use of the latter as read-outs of muscle metabolism.

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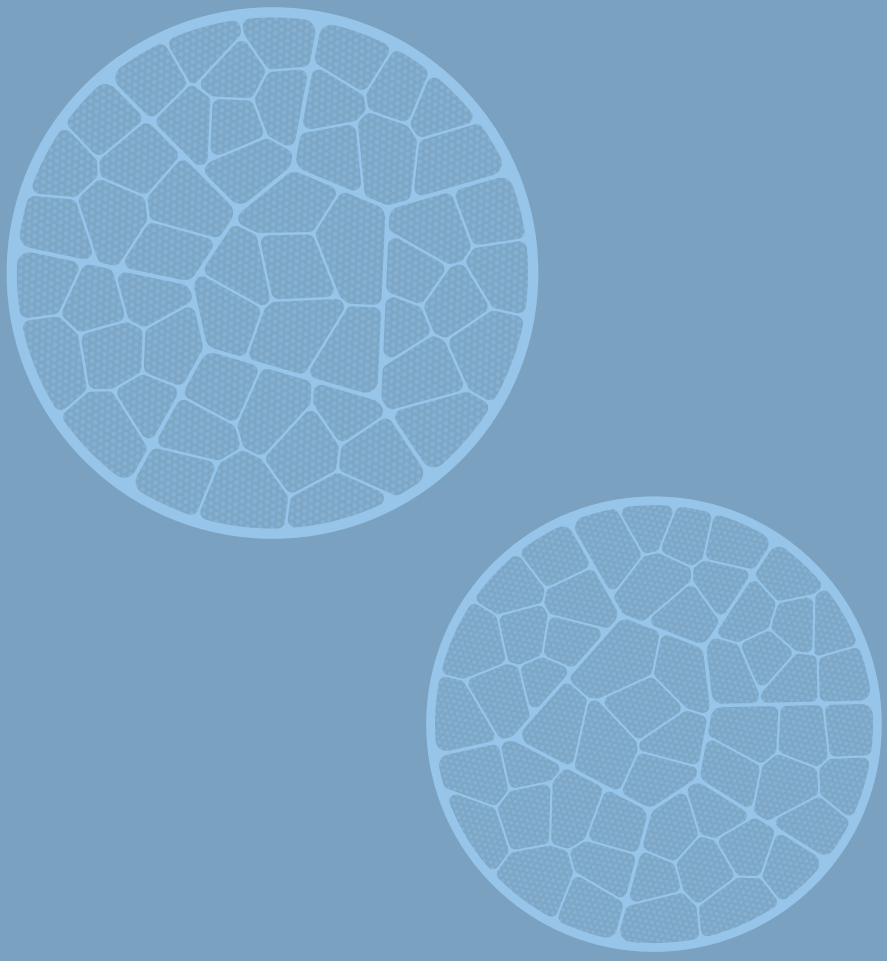
Chapter 4 - Creatine supplementation attenuates changes in skeletal muscle transcriptome during knee immobilization



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In preparation

Abstract

Knee immobilization can induce rapid loss of muscle mass and strength in humans. We assessed the effect of knee immobilization with creatine supplementation or placebo on the skeletal muscle transcriptome and metabolome.

Sixteen healthy young men underwent knee immobilization for 7 days using a full-leg cast. Subjects received 20g creatine or a placebo supplement per day for 5 days prior to immobilization, followed by 5g creatine or placebo per day during immobilization. Muscle biopsies were obtained from the *vastus lateralis* prior to immobilization and immediately after cast removal and subjected to transcriptomics. Metabolomics were performed on a subset of the muscle biopsies (12 subjects in total, 6 subjects per group).

Knee immobilization caused muscle mass loss (-574 ± 115 mm^2 whole leg cross-sectional area, $P < 0.01$) and strength loss (-7.4 ± 1.8 kg leg extension 1RM, $P < 0.01$) in all participants, with no differences between creatine and placebo groups. Knee immobilization significantly induced expression of ubiquitin-mediated proteolysis genes, whereas glucose metabolism and mitochondrial genes were downregulated. Gene expression changes due to immobilization tended to be smaller in the creatine group. Genes belonging to the HDAC4-Myogenin axis were significantly upregulated, which was attenuated by creatine supplementation. Knee immobilization induced a significant decrease in several metabolites including fatty acid derived acyl carnitines, putrescine, ornithine, proline and lactic acid.

Knee immobilization appears to induce the HDAC4-myogenin axis, which is primarily associated with denervation and motor neuron diseases. Transcriptional changes in skeletal muscle due to knee immobilization were attenuated by creatine supplementation. The metabolome showed changes consistent with the decreased expression of energy metabolism genes.

Introduction

Brief periods of physical inactivity can have a dramatic negative effect on skeletal muscle mass and function in humans. In addition to the rapid loss of muscle strength and size, secondary negative effects on the muscle may ensue, including a decrease in insulin sensitivity, loss in the capacity to synthesize protein, decline in basal metabolic rate, and gain of fat mass [1-5]. As a consequence, limb immobilization (e.g., due to a fracture) can have negative effects in for example athletes, but also older adults who are more vulnerable to muscle loss. Thus, novel strategies to combat the negative effects of brief periods of physical inactivity are in great demand.

Creatine supplementation has been used as a strategy to augment gains in muscle mass and strength, particularly by athletes in strength and power sports. Creatine supplementation can increase the concentration of creatine phosphate in skeletal muscle, thereby raising the rapidly available energy supply in the muscle [6-10]. This larger supply of energy allows for a higher training load, potentially leading to improved strength and muscle gains during prolonged resistance-type exercise training. Creatine supplementation may also have secondary benefits that may influence muscle hypertrophy and muscle strength gains. For example, it draws water into the muscle cells, which is thought to modulate muscle signalling [11]. It is common to see rapid weight gain after initiation of creatine supplementation, due to muscle cell swelling [12, 13].

Given the anabolic potential of creatine supplementation, several studies have evaluated the use of creatine supplementation as a tool to limit the loss of muscle mass and strength during limb immobilization. However, the results of these studies are thus far inconsistent. Two studies observed an attenuation of muscle losses during immobilization in the creatine group, one in rats and one in humans [14, 15]. We were, however, unable to replicate these results [16]. Another study also investigated whether creatine supplementation combined with resistance-type exercise training after a period of immobilization could improve recovery time. It was observed that creatine supplementation indeed had a positive effect on recovery time and modulated regulatory factors in the muscle. However, no beneficial effect of creatine with regard to muscle mass and strength losses was observed during immobilization [17].

Because of the wide and pronounced effects immobilization can have on both muscle size and metabolism we were interested in investigating both the muscle transcriptome and metabolome in relation to both knee immobilization and creatine supplementation during this period of muscle disuse. Transcriptomics and metabolomics allow us to characterize in more detail the transcriptional and metabolic changes that occur during immobilization. These techniques may also reveal the more subtle effect creatine supplementation has on the skeletal muscle transcriptome, especially given that creatine supplementation appears to have an effect on regulatory factors and cell metabolism. In short, our aim is to investigate

genes, metabolites and pathways involved in immobilization-induced muscle atrophy and to determine whether this response can be modulated by creatine supplementation.

Methods

Experimental design and procedures

This study used a subset of participants from a larger randomized, double-blind placebo controlled study. More detailed methods and materials can be found elsewhere [16]. Subjects were randomly allocated into either the placebo (n=8) or the creatine (n=8) supplemented group. Standardized meals were consumed the evening prior to the muscle biopsies. All measurements, with exception of the strength measurements, were taken in a fasted state.

A muscle biopsy was taken and muscle size (cross-sectional CT scan of the upper legs) and strength (1RM leg extension) was measured two days prior to immobilization. One leg was immobilized using a full leg cast for seven subsequent days. On the day of the cast removal, another muscle biopsy was taken and muscle size and strength measurements were repeated. Microarrays and metabolomics analyses were performed on the muscle biopsies. The subset of subjects was based on muscle tissue availability for all time points. A sufficient amount of muscle tissue for the metabolomics analysis was available for 12 subjects (n=6 for both groups) and 16 subjects for the transcriptomics analysis (n=8 for both groups).

Muscle mass was assessed with a single slice CT-scan (Philips Brilliance 64, Philips Medical Systems, Best, the Netherlands). Scan position was marked for to ensure accurate repeated measurements. Maximal leg extension strength was estimated using unilateral single repetition maximum (1RM) tests for both legs. After warming up, subjects were asked to take place on a leg extension machine (Technogym, Rotterdam, The Netherlands). Subjects were then asked to perform leg extensions using strict form. Weight was increased until the subject could no longer lift the weight. During the screening visit, sub-maximal strength was approximated using multiple repetition maximum to ensure a starting weight close to the true 1RM for the strength measurements. The highest weight that was successfully lifted was used as 1RM.

Limb immobilization

One randomly selected leg was immobilized at the Maastricht University Medical Centre. The leg was cast at a 30° angle to prevent weight bearing activities of the immobilized leg. Subjects were given crutches for the duration of the leg immobilization period. Subjects were instructed to perform daily ankle exercises to minimize risk of thrombosis via activation of the calf pump. After seven days of immobilization the cast was removed at the hospital. Following cast removal subjects were transported by wheelchair to prevent any muscle loading prior to muscle biopsies.

Subjects

This study included sixteen healthy, young men. Exclusion criteria included history or family history of thrombosis, any joint complaints and any co-morbidities interacting with mobility or muscle metabolism of the lower limbs. None of the subjects had performed strength training over the past half year, used anti-coagulants, corticosteroids, growth hormone, testosterone, immunosuppressant drugs or exogenous insulin. During the screening, subjects gave written informed consent after they were fully informed about the nature and possible risks of the experimental procedures. The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre+ and performed in accordance with the Declaration of Helsinki.

Muscle Biopsy

Body weight was measured with a digital balance with an accuracy of 0.1 kg (SECA GmbH, Hamburg, Germany). Muscle biopsies were taken from the middle region of the *vastus lateralis* muscle under local anaesthesia, ~15 cm above the patella and ~3 cm below entry through the fascia, using the percutaneous needle biopsy technique [18]. All biopsies were taken from the leg that was subsequently immobilized. Muscle samples were dissected carefully and freed from any visible non-muscle material and were immediately frozen in liquid nitrogen. Subsequently, muscle samples were stored at -80 °C until further analysis.

Supplements and supplementation protocol

The placebo group received a placebo supplement consisting of 7.5 g maltodextrin and 7.5 g of dextrose monohydrate (AVEBE, Veendam, The Netherlands). The creatine group received the same supplement with the addition of 5 g creatine monohydrate (Creapure AlzChem, Trostberg, Germany). Creatine supplements could not be distinguished from the placebo supplement with respect to colour, taste, smell or appearance. All subjects were instructed to dissolve the supplement in lukewarm water prior to ingestion. During the first five days of creatine loading, subjects consumed four supplements per day (providing a total of 20 g creatine per day for the creatine group) at breakfast, lunch, dinner and before going to bed. After the five day loading period subjects consumed one supplement per day for the remainder of the study. This protocol was designed according to the guidelines on creatine supplementation set by the American College of Sports Medicine [13].

Microarray analysis

RNA was isolated (RNeasy Micro kit, Qiagen, Venlo, the Netherlands), quantified (Nanodrop ND 1000, Nanodrop technologies, Wilmington, DE, USA) and integrity was checked by an Agilent 2100 Bioanalyser with RNA 6000 microchips (Agilent Technologies, South Queensferry, UK). Total RNA was labelled using the GeneChip® WT plus Reagent Kit and hybridized to GeneChip® Human Gene 1.1 ST Array (Affymetrix, Inc. Santa Clara, CA, USA). Sample labelling,

hybridization to chips, and image scanning were performed according to the manufacturers' instructions.

Metabolome analysis

Metabolites were extracted from 10 mg of wet muscle tissue. This tissue was further lyophilized and weighted to determine the dry tissue mass. After pulverizing the tissue, metabolites were extracted using methanol/chloroform/water (MCW). The extraction method used in this study has extensively been described and characterized elsewhere [19]. Amino acids and biogenic amines in plasma were derivatized (AccQ-TAG) 5 µL aliquots of muscle extract and measured by a UPLC system which was interfaced to quadrupole mass spectrometer. Acylcarnitines, trimethylamine-N-oxide, choline, betaine, deoxycarnitine and carnitine were analyzed in 5 µL muscle extract, spiked with an internal standard and also measured by UPLC-MS, but without derivatisation. Organic acids in plasma were measured by GC-MS, after oximation and silation derivatization. Full details of these platforms have been described in earlier studies [19, 20].

Data analysis

Physical characteristics (i.e. muscle size and strength) were evaluated using linear mixed models. Microarray data were assessed for quality using the MADMAX pipeline and additionally by visually inspecting the probe level residuals and NUSE plots [21]. Data was normalized using Robust Multichip Average (RMA) [22]. Gene level summarization was performed using the most recent Custom CDF from the Brainarray project [23]. Genes were filtered using UPC filtering with a 50% expression likelihood cut-off in at least 8 samples [24].

Statistical analysis was performed using the *limma* R library [25]. Contrasts were set for immobilization effect in both groups and an interaction term for the creatine group versus the placebo group. P-values were calculated using Intensity Based Moderated t-tests (IBMT) [26]. Significant genes were first selected using the False Discovery Rate adjusted F-statistic p-value < 0.05. Unadjusted p-values below 0.01 for the contrasts were considered statistically significant within the genes that passed the F-test. Gene set enrichment analysis (GSEA) was done using the *piano* R library on pre-ranked lists, ranked by the t-values from the *limma* contrasts [27]. We used the most recent library of canonical pathways from MsigDb [28]. A q-value of 0.10 was considered significant for the GSEA results. Missing data in the metabolome were imputed by taking half the lowest value for that metabolite (5 values in total, 1 for cytidine triphosphate, 3 for uridine triphosphate and 1 for valerylcarnitine). Data was $\log_2$ transformed prior to analysis. Statistical analysis of metabolome was done using paired t-tests (post vs. pre-immobilization) for each metabolite. Data visualization was done using *ggplot2* and *gplots* [29, 30].

Results

Immobilization effect

Seven days of knee immobilization induced rapid loss of muscle mass. Leg CSA was reduced by 574 ± 115 mm^2 and quadriceps CSA was reduced by 484 ± 65 mm^2. These changes were accompanied by decreased maximal strength, as revealed by a reduction in leg extension 1RM of 7.4 ± 1.8 kg. Creatine supplementation did not have a significant effect on the loss of both muscle mass and muscle strength (table 4.1, page 78). A full discussion of the physiological effects of knee immobilization with or without creatine supplementation has been previously published [16].

Knee immobilization induced significant changes in the muscle transcriptome in both the placebo group and the creatine group, with 520 genes significantly affected in total (F-test q-value < 0.05). Fewer genes were altered by knee immobilization in the creatine group than in the placebo group (229 vs. 439, respectively). Table 4.2 shows the top 20 significant genes based on fold change in the placebo group. Supplementary table 1 contains all significant genes according to the main effect F-test.

Table 4.1 – Baseline characteristics and physiological effects of the intervention. Values are unadjusted means ± standard deviation. Creatine levels are in mmol per kg dry muscle. P-values are from linear mixed models. Immob. is the effect of immobilization (pre vs. post). Group is treatment group (creatine or placebo). BMI = body mass index, CSA = cross-sectional area, 1RM = 1 repetition maximum, *** = P < 0.001, ** = P < 0.01, * = P < 0.05, † = P < 0.10.

	Creatine - Pre	Creatine - Post	Placebo - Pre	Placebo - Post	Immob.	Group	Immob. : Group
Age (years)	22.5 ± 3.5		22.9 ± 3.2				
Height (m)	1.80 ± 0.10		1.80 ± 0.10				
Weight (kg)	73.2 ± 8.4		71.4 ± 14.5				
BMI (kg / m²)	22.7 ± 1.7		23.1 ± 3.3				
Leg CSA (mm²)	14841 ± 1250	14267 ± 1248	14510 ± 2004	14004 ± 1981	0.000***	0.756	0.684
Quadriceps CSA (mm²)	8259 ± 953	7776 ± 927	7844 ± 1363	7428 ± 1313	0.000***	0.557	0.475
Fat CSA (mm²)	3390 ± 797	3363 ± 813	4128 ± 2154	4175 ± 2167	0.451	0.336	0.156
Leg Extension 1RM (kg)	60 ± 10	52 ± 9	53 ± 15	52 ± 17	0.001**	0.781	0.087†
Creatine (mmol / kg)	53.7 ± 6.4	47.0 ± 12.5	47.5 ± 10.3	47.0 ± 11.5	0.169	0.998	0.365
Phosphocreatine (mmol / kg)	44.6 ± 19.6	56.3 ± 21.1	41.7 ± 14.9	39.8 ± 14.1	0.164	0.072†	0.247
Total Creatine (mmol / kg)	98.3 ± 18.4	103.3 ± 16.4	89.2 ± 11.6†	86.7 ± 9.9	0.449	0.031*	0.422

Table 4.2 – Top 20 significant genes in the placebo group sorted on absolute signal log ratio. P-values for the immobilization effect in the creatine and placebo group as well as the interaction effect of creatine with immobilization are unadjusted IMBT p-values. FC is the signed fold change. Cre is the change in the creatine group, Pla is the change in the placebo group and Inter is the interaction effect between creatine supplementation and immobilization.

Gene	FC Pla	FC Cre	P-val. Pla	P-val. Cre	P-val. Inter	Q-val. F-test
NMRK2	-5.23	-2.00	0.000	0.011	0.013	0.003
SMCO1	-4.66	-2.75	0.001	0.015	0.331	0.016
GDNF	4.64	2.56	0.000	0.003	0.136	0.004
ACTN3	-4.38	-1.58	0.001	0.210	0.057	0.029
NR4A1	-3.73	-1.43	0.000	0.174	0.015	0.010
HDAC4	3.24	1.67	0.000	0.006	0.010	0.002
DHRS7C	-3.14	-2.26	0.003	0.026	0.498	0.036
AMPD3	2.84	2.28	0.004	0.017	0.625	0.033
GADD45A	2.68	2.52	0.008	0.012	0.894	0.040
SLC16A3	-2.65	-1.74	0.000	0.003	0.077	0.004
LRRC14B	-2.65	-1.53	0.002	0.125	0.163	0.045
TP63	2.62	1.37	0.000	0.068	0.012	0.006
COQ10A	-2.48	-2.08	0.001	0.007	0.607	0.016
TFRC	-2.45	-2.19	0.000	0.001	0.709	0.007
AKR1C2	2.43	1.50	0.000	0.050	0.096	0.014
PFKFB3	2.41	2.70	0.007	0.003	0.783	0.022
IP6K3	2.32	1.73	0.000	0.000	0.102	0.002
FAM46C	-2.32	-1.24	0.000	0.154	0.007	0.006
ASB14	-2.32	-1.08	0.001	0.697	0.015	0.035
CCNG2	2.30	1.78	0.000	0.008	0.366	0.012

Among the top 20 significant genes for the placebo group are genes related to energy metabolism, including Nicotinamide Riboside Kinase 2 (NMRK2), Solute Carrier Family 16, Member 3 (Monocarboxylic Acid Transporter 4; SLC16A3), 6-Phosphofructo-2-Kinase/ Fructose-2,6-Biphosphatase 3 (PFKFB3), Inositol Hexakisphosphate Kinase 3 (IP6K3) and Nuclear Receptor Subfamily 4 Group A Member 1 (NR4A1 / Nur77) (table 4.2). Neurological function genes were also among the top genes changed by knee immobilization, including Histone Deacetylase 4 (HDAC4), Glial Cell Derived Neurotrophic Factor (GDNF), Maturin, Neural Progenitor Differentiation Regulator Homolog (MTURN) and Ataxin 1 (ATXN1).

Consistent with these top genes are the results from GSEA. GSEA revealed downregulation of glycolysis, gluconeogenesis and mitochondrial function pathways (table 4.3, supplementary table S4.2). GSEA also revealed increased expression of proteasomal genes and ubiquitin mediated proteolysis pathways. However, two markers of muscle breakdown, TRIM63 and FBXO32 (Better known as MURF1 and MAF-bx/Atrogin-1, respectively)

were not strongly affected by the immobilization despite the upregulation of ubiquitin mediated proteolysis genes and proteasomal genes (table 4.2, supplementary table S4.1). Nevertheless, a regulator of muscle atrophy, Growth Arrest And DNA Damage Inducible Alpha (GADD45A), is among the top genes (table 4.2). Upstream regulator analysis using Ingenuity showed that knee immobilization led to decreased expression of insulin target genes and increased expression of Forkhead box O3 (FOXO3), DNA-binding protein inhibitor ID2 and ID3 target genes, indicative of the decreased expression of glucose metabolism genes and increased expression of atrophy genes, respectively (table 4.4).

Table 4.3 – Top 20 significant enriched genesets (GSEA) in significant in both the placebo arm and the effect of creatine supplementation during immobilization (interaction effect). Pla is the immobilization effect, Inter is the interaction effect. ES is the enrichment score; negative ES denotes downregulation.

Pathway	*Pla ES*	*Inter ES*	*Pla Q-val*	*Inter Q-val*
Reactome Unwinding Of DNA	-0.80	0.72	0.000	0.015
Biocarta SRCRPTP Pathway	-0.75	0.63	0.004	0.053
Biocarta Glycolysis Pathway	-0.74	0.70	0.041	0.055
Biocarta Monocyte Pathway	-0.72	0.61	0.014	0.065
Reactome Gluconeogenesis	-0.71	0.46	0.000	0.094
Biocarta B-lymphocyte Pathway	-0.70	0.63	0.017	0.057
Reactome Pyrimidine Catabolism	-0.70	0.60	0.018	0.079
PID IL8 CXCR2 Pathway	-0.68	0.68	0.000	0.000
Reactome Pol Switching	-0.67	0.77	0.024	0.006
Biocarta TCRA Pathway	-0.67	0.58	0.036	0.091
PID IL8 CXCR1 Pathway	-0.67	0.65	0.000	0.000
Reactome PD1 Signaling	-0.66	0.55	0.004	0.060
Biocarta IL5 Pathway	-0.65	0.64	0.064	0.073
Reactome Regulated Proteolysis Of P75NTR	-0.64	0.84	0.071	0.006
PID Alpha-Synuclein Pathway	-0.64	0.59	0.000	0.003
Biocarta Lym Pathway	-0.63	0.66	0.074	0.059
Biocarta Barrestin Src Pathway	-0.63	0.53	0.025	0.094
Reactome Signaling By Notch2	-0.63	0.76	0.054	0.011
Reactome Regulation Of Complement Cascade	-0.63	0.59	0.055	0.089
Reactome P38MAPK Events	-0.63	0.60	0.051	0.059

Effect of Creatine Supplementation

Figure 4.1 shows the change in gene expression for each during leg immobilization in both the placebo group and creatine group. The creatine group did not show a strong distinct effect on the transcriptome and in general the response was correlated with the placebo group. However, the magnitude of the change in gene expression was generally lower in the creatine group, as shown in table 4.2 and figure 4.1. The shaded area in figure 4.1 covers the genes whose expression change was attenuated in the creatine group compared to the placebo group. GSEA showed a similar result, as gene sets that were significant for knee immobilization were predominantly counter-regulated by creatine supplementation (table 4.3, supplementary table 4.2 and 4.3). Upstream analysis also showed that creatine supplementation modulated Myocyte Enhancement Factor 2C (MEF2C) target genes during immobilization (table 4.4).

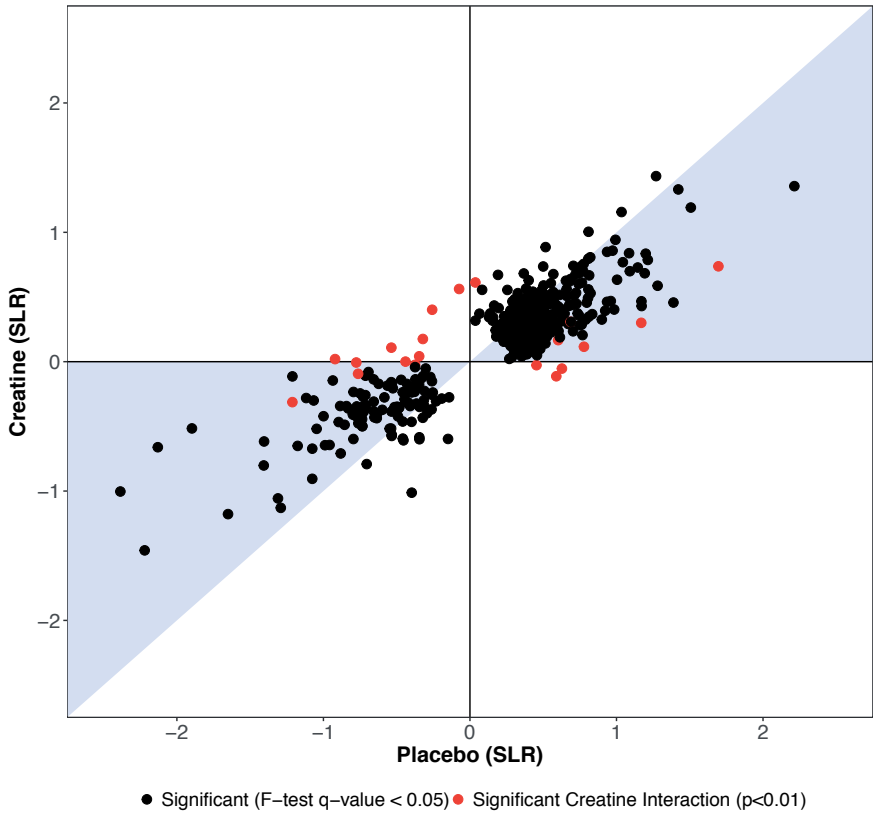

Figure 4.1 – Signal log ratios of significant genes during immobilization (placebo group vs. creatine group). All genes are significant based on the overall ANOVA F-test (q-value < 0.05). Red points are considered genes significantly affected by creatine supplementation (raw p-value for interaction < 0.01). Shaded area shows genes where the change in gene expression is smaller in the creatine group compared to the change in the placebo group.

Table 4.4 – Top 10 upstream regulators from Ingenuity Pathway Analysis.

Upstream Regulator	p-value of overlap	Activation z-score
Immobilization - Placebo group		
ID2	0.000	0.302
ID3	0.001	-0.560
ALKBH5	0.002	
FOXO3	0.002	2.084
IFI16	0.003	0.810
Insulin	0.003	-1.868
SPIB	0.005	0.378
MAP2K5	0.005	
HDAC4	0.006	0.842
Immobilization - Creatine group		
ITSN1	0.003	
FOXD1	0.003	
NFE2L2	0.005	3.075
RORC	0.006	
ONECUT1	0.007	
GPD1	0.009	
SLC25A13	0.009	
RARRES3	0.010	
Foxo	0.019	
HCK	0.019	
Creatine Interaction		
FNIP1	0.000	-2.200
PDGF BB	0.000	-1.997
FGF10	0.001	1.276
ZFPM1	0.001	
STAT5B	0.001	-1.497
ETV7	0.002	
MEF2C	0.002	2.614
TAF4B	0.003	
DIO2	0.003	

Changes in Metabolome during Immobilization

Most metabolite levels showed decreased levels after immobilization, regardless of significance (figure 4.2). Predominant among the significant metabolites (p-value < 0.05) were fatty-acid derived acyl carnitines, including malonylcarnitine, dodecenoylcarnitine, acetylcarnitine and decenoylcarnitine. These carnitines all showed decreased levels after immobilization. A single carnitine, 2-methylbutyroylcarnitine, a product of branched chain amino acid oxidation, showed increased levels after immobilization. Two metabolites from polyamine metabolism, ornithine and putrescine, also showed decreased levels after immobilization. Two other metabolites in the polyamine pathway, spermine and spermidine, did not pass quality control. Lactic acid and GABA levels were also decreased after immobilization. No significant effects of creatine supplementation on the metabolome were observed.

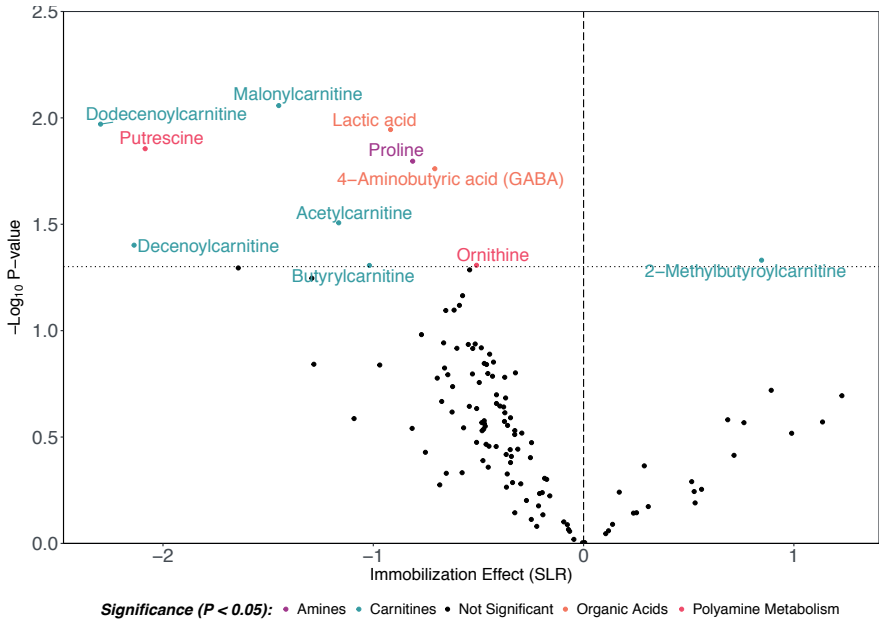

Figure 4.2 – Volcano plot of metabolites changed during immobilization. Metabolites are coloured by type. Black points are metabolites that were not significant (p-value < 0.05). Dotted line represents a p-value cut-off of 0.05.

Discussion

Knee immobilization rapidly reduced muscle mass and strength. Contrary to our hypothesis, creatine supplementation did not attenuate loss of muscle mass and strength by knee immobilization. However, we observed that changes in the skeletal muscle transcriptome were attenuated in the creatine group as compared to the placebo group, despite a lack of significant differences in muscle size and strength loss. Analysis of the response in the skeletal muscle metabolome during immobilization revealed a decrease in fatty-acid derived acyl-carnitines and two metabolites involved in polyamine metabolism. We did not observe any differences due to creatine supplementation in the metabolome, likely due to the low sample size, and high inter-individual variation in the metabolomics data.

Previous studies have observed that short-term physical inactivity can lead to decreased insulin sensitivity and anabolic resistance [3, 4, 31]. Our findings are consistent with these previous observations, which are likely due to the decreased energy demands of inactive skeletal muscle. Several downregulated genes among the top 20 genes are involved in energy metabolism, including NMRK2, SLC16A3, PFKFB3 and NR4A1 (table 4.2). The latter, NR4A1 (frequently referred to as Nur77), is a major regulator of glucose metabolism in muscle tissue [32-34]. The results of gene set enrichment analysis are consistent with the decreased expression levels of these energy metabolism genes, showing decreased expression of mitochondrial function genes and glucose metabolism genes (table 4.3).

The decreased levels of several fatty acid derived acyl-carnitines also imply a decreased turnover of fatty acids. These shifts in expression of genes related to energy metabolism and the decrease in fatty acid derived acyl-carnitines are likely also due to the significant reduction in energy demand during immobilization. The only metabolite that was increased after immobilization was 2-methylbutyroylcarnitine, which is a downstream product of branched chain amino acid oxidation. The most likely explanation is increased amino acid oxidation due to muscle protein breakdown.

We observed a reduction in the levels of putrescine and ornithine. Putrescine is a metabolite that is part of polyamine metabolism, while the conversion of ornithine to spermine is the rate limiting step in polyamine synthesis. Unfortunately, spermine and spermidine, the two other main polyamines in polyamine metabolism, did not pass quality control. The polyamine pathway regulates a wide range of metabolic processes, including cell cycle and growth pathways [35-37]. Importantly, it is also involved in muscle health, where it is associated with both muscle growth and muscle atrophy [38, 39]. Previous evidence suggests that putrescine is increased in denervated muscle in rats [40]. Furthermore, unloading via a hind-limb suspension model impacts muscle polyamine levels in mice, although the effects appear to be different between fast-twitch and slow-twitch muscle and mainly concern spermine and spermidine [39, 41, 42]. Unfortunately, there are very few data on the role of polyamine metabolism in human skeletal muscle.

Both Histone Deacetylase 4 (HDAC4) and Myogenin were significantly upregulated after immobilization, whereas HDAC9 was downregulated. The HDAC4/Myogenin axis involves HDAC4, HDAC9, Myogenin and Myocyte Enhancement Factor 2 (MEF2) and has been primarily studied in the context of neurogenic muscle atrophy [43, 44]. HDAC4 interacts with Myogenin and together these factors regulate muscle atrophy genes [45]. MEF2 is a major regulator of skeletal muscle gene expression and has been shown to be inhibited by HDAC4 [46]. Even though there is no denervation or neuromuscular pathology in these healthy volunteers, it may be that the chronic lack of muscle contraction leads to a similar lack in calcium fluxes in the sarcoplasm, thus inducing a similar muscle programme that regulates both muscle atrophy and neurogenesis. Previous studies have shown that HDAC4 and Myogenin are also involved in diseases with a neuromuscular component such as Huntington's and Amyotrophic Lateral Sclerosis [47-51].

Immobilization led to differential expression of several other genes that play a role in neuromuscular signalling, which seems to be consistent with the increased expression of HDAC4 and Myogenin. Glial cell derived neurotrophic factor (GDNF), involved in motor neuron survival and regeneration, was significantly upregulated and features among the top 20 differentially expressed genes (table 2). Muscle cholinergic receptor subunits were all upregulated, although only the B1 subunit (CHRNB1) reached statistical significance (supplementary table 1). Lastly, acetylcholine esterase was significantly downregulated (supplementary table 1). Ataxin-1 (ATXN1) was also significantly upregulated, which is known to interact with HDAC4 and MEF2 and plays a central role in spinocerebellar ataxia 1 [52]. In aggregate these gene expression changes suggest a compensatory response to restore neuronal signalling via increased expression of muscle acetylcholine receptors, enhanced motor neuron survival via GDNF, and decreased breakdown of acetylcholine at the neuromuscular junction.

Gene expression changes due to immobilization were smaller in the creatine group than in the placebo group (table 4.2, figure 4.1, supplementary table S4.1). It is unknown how creatine can modulate the transcriptomic response to knee immobilization. A possible indirect factor could be activation of p38 MAPK by creatine supplementation. Previous studies have shown that creatine supplementation increases expression of p38 MAPK and induces activation of p38 MAPK, likely via osmotic stress [53, 54]. Furthermore, the p38 MAPK pathway has previously been shown to modulate MEF2 activity [55, 56]. Another mechanism could be the upregulation of basic helix-loop-helix e40 (BHLHE40). This gene was upregulated in the creatine group (FC 1.48, p-val. 0.003) but unaffected in the placebo group (supplementary table S4.1). Previous studies have shown that BHLHE40 can repress the activity of myogenic factors, including MyoD and Myogenin [57, 58]. It is possible that the induction of BHLHE40 mediates the attenuating effect of creatine supplementation on the transcriptomic response since Myogenin plays an important role in HDAC4-induced muscle atrophy. Additionally, the study by Hespel et al. also showed that creatine supplementation modulated Myogenin protein levels after knee immobilization [17].

In contrast to the observed effect of creatine on the skeletal muscle transcriptome, we were unable to find an effect of creatine supplementation on muscle size and strength. It is possible that the effects of creatine supplementation will start to manifest during longer term knee immobilization, as is the case with a fracture. Of course, gene expression does not necessarily translate directly to protein expression and as such these results should be interpreted with caution. However, these results do suggest that it might be worthwhile to investigate whether the lack of effect of creatine supplementation is due to the brief period of immobilization or whether there is a true lack of effect of creatine supplementation on muscle atrophy.

Conclusion

Short term knee immobilization led to decreased expression of genes involved in energy metabolism and seemed to induce muscle atrophy via the HDAC4/Myogenin axis. This mechanism has previously been studied primarily in the context of denervation. In humans HDAC4 activity in skeletal muscle is primarily observed in disease states such as ALS and Huntington's, whereas here it is induced in healthy human volunteers. This suggests that knee immobilization can be used as a model to study the HDAC4/Myogenin axis in healthy humans. Metabolomics showed alterations in the polyamine metabolism, suggesting involvement of polyamine metabolism in muscle disuse atrophy. Creatine supplementation seemed to attenuate gene expression changes due to knee immobilization but this result did not translate into a measurable effect on changes in muscle size and strength.

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Conflict of interest

John van Duynhoven is employed by a company that manufactures and markets food products. The other authors have no competing interests to declare.

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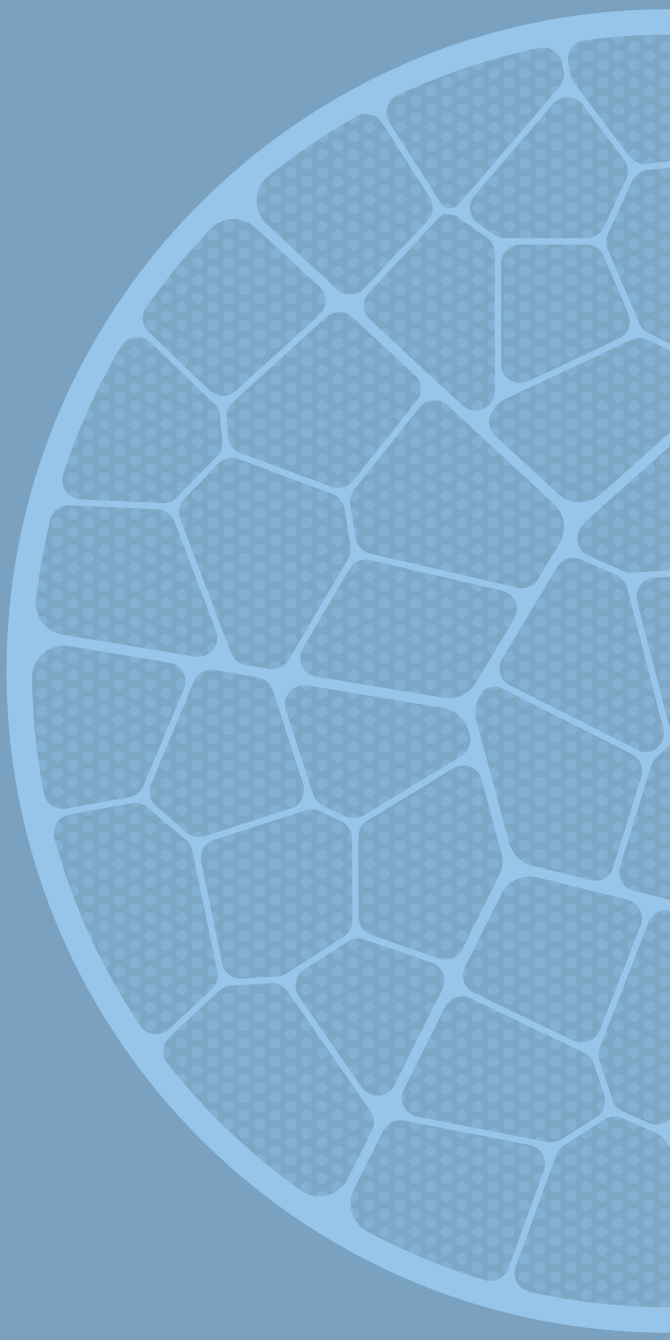
Chapter 5 - No effect of calcifediol supplementation on skeletal muscle transcriptome in vitamin D deficient frail older adults



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Abstract

Vitamin D deficiency is common among older adults and has been linked to muscle weakness. Vitamin D supplementation has been proposed as a strategy to improve muscle function among older populations. We investigated the effect of calcifediol on the skeletal muscle transcriptome in vitamin D deficient (25(OH)D between 20 and 50 nmol/L) frail older adults (65+) in a double-blind placebo controlled trial.

Twelve subjects were included in the placebo group and ten subjects were included in the calcifediol group. Muscle biopsies were obtained before and after six months of calcifediol (10 µg per day) or placebo supplementation. Whole genome gene expression profiling was done using Affymetrix HuGene 2.1ST arrays.

Calcifediol supplementation led to a significant increase in blood 25(OH)D levels compared to the placebo group. No difference between treatment groups was observed on strength outcomes. P-values were uniformly distributed across all genes, suggesting that low p-values are likely to be false positives. PLS-DA was unable to separate treatment groups. Significant genes did not reveal pathways associated with muscle function or vitamin D signalling.

We did not observe a robust effect of calcifediol supplementation on the skeletal muscle transcriptome among frail older adults. Our findings indicate the effects of vitamin D supplementation on skeletal muscle may be either absent, weak, or limited to a subset of muscle cells.

Introduction

Muscle weakness and muscle loss increase with age, potentially leading to an increased risk of falls, frailty and loss of independence among older adults [1]. Many older adults are also deficient in vitamin D. Various observational studies have found associations between vitamin D deficiency and impaired muscle function and/or physical performance among older populations [2-6]. As a consequence, vitamin D supplementation has been proposed as a strategy to improve muscle function among older populations.

Vitamin D is suspected to affect muscle health via both indirect and direct mechanisms. Indirectly vitamin D can influence muscle function via its role in calcium and phosphate homeostasis [7, 8]. Vitamin D has also been proposed as a direct modulator of skeletal muscle signalling via activation of the vitamin D receptor. Several cell culture studies have suggested a role of vitamin D receptor signalling in skeletal muscle health and function [9-12]. In addition, inactivation of the vitamin D receptor in mice leads to impaired muscle development and differential expression of key myogenic regulators [13]. Conversely, calcitriol was found to inhibit myoblast proliferation and differentiation in primary myocytes isolated from human skeletal muscle [14].

While there is evidence from *in vitro* studies and animal knock-out models suggesting a transcriptional role of the vitamin D receptor in skeletal muscle, more recently this role has come into question [15]. Wang et al. (2011) showed that most of the antibodies for the vitamin D receptor lacked specificity, potentially leading to false positives. Intriguingly, despite the fact that the vitamin D receptor was present and functional in myocytes isolated from human skeletal muscle and C2C12 myotubes, Olsson and colleagues (2016) were unable to detect appreciable levels of the vitamin D receptor in mature human skeletal muscle. We performed RNA microarray measurements on muscle biopsies obtained from frail older participants of a randomized, placebo controlled double blind trial investigating the effect of calcifediol (25-hydroxycholecalciferol or 25(OH)D) supplementation on muscle function [16]. Our goal was to determine the overall impact of vitamin D on skeletal muscle gene regulation *in vivo* in humans and identify potential target genes in skeletal muscle.

Materials & Methods

Study Design & Population

This study is part of a larger clinical trial. Procedures for this study will be described briefly, more details can be found elsewhere [16]. This study used a randomized, parallel-arm double blind design. Subjects in the calcifediol arm received 10ug 25(OH)D per day (DSM Nutritional Products Ltd.). Subjects were instructed to take their capsules in the morning during breakfast. A subset of samples was taken from the main study based on how much muscle was available for microarray analysis (12 subjects in the placebo arm and 10 subjects

in the calcifediol arm). Participants were frail and pre-frail older adults (65+) with serum levels of 25(OH)D between 20-50nmol/L. Frailty was assessed using the Fried criteria [17].

Strength Measurements

Isometric leg muscle strength (leg extension and leg flexion) was assessed using a Biodex System 4 dynamometer (Biodex Medical Systems, Shirley, NY, USA). Subjects were seated upright with their chest and waist secured by belts. Experiments were performed with knee angle of 60^{0} and hip angle of 90^{0}. Subjects performed 3 maximal voluntary isometric contractions for five seconds, with 30 seconds of rest between trials and five minutes of rest between knee-extension and knee-flexion trials. Researchers provided standardized verbal encouragement during the strength tests.

Muscle Biopsies

Muscle biopsies were taken from the middle region of the *vastus lateralis* muscle under local anaesthesia, ~15 cm above the patella and ~3 cm below entry through the fascia, using the percutaneous needle biopsy technique [18]. Muscle samples were dissected carefully and freed from any visible non-muscle material and were immediately frozen in liquid nitrogen. Subsequently, muscle samples were stored at -80 °C until further analysis.

Microarray analysis and qPCR

RNA was isolated (RNeasy Micro kit, Qiagen, Venlo, the Netherlands), quantified (Nanodrop ND 1000, Nanodrop technologies, Wilmington, DE, USA) and integrity was checked by an Agilent 2100 Bioanalyser with RNA 6000 microchips (Agilent Technologies, South Queensferry, UK). Total RNA was labelled using the GeneChip® WT plus Reagent Kit and hybridized to GeneChip® Human Gene 2.1 ST Array (Affymetrix, Inc. Santa Clara, CA, USA). Sample labelling, hybridization to chips, and image scanning were performed according to the manufacturers' instructions.

We performed qPCR on the vitamin D receptor gene (forward: GTGGACATCGGCATGATGAAG, reverse: GGTCGTAGGTCTTATGGTGGG). 500ng RNA was reverse transcribed to cDNA using a iScript cDNA synthesis kit (Bio-Rad Laboratories, Veenendaal, Netherlands). Real-time PCR was performed using SensiMix (Bioline, GC biotech, Alphen aan den Rijn, Netherlands) on a CFX384 Real-Time PCR detection system (Bio-Rad Laboratories, Veenendaal, Netherlands).

Data analysis

All data analysis was done in *R* [19]. Changes in muscle strength and vitamin D levels were evaluated using linear mixed models using the *lme4* library[20]. Microarray data were assessed for quality using the MADMAX pipeline and additionally by visually inspecting the probe level residuals and NUSE plots[21]. Data was normalized using Robust Multichip Average (RMA) (Bolstad et al., 2003). Gene level summarization was performed using the most

recent Custom CDF from the Brainarray project [22]. Genes were filtered using UPC filtering with a 50% expression likelihood cut-off in at least 10 samples, the smallest subset within this dataset [23].

Univariate statistical analysis of gene expression was performed using the *limma* R library (Ritchie et al., 2015). Contrasts were set for time effect in both placebo and calcifediol groups and an interaction term for the calcifediol group versus the placebo group. P-values were calculated using Intensity Based Moderated t-tests[24]. Genes with a p-value below 0.05 and an absolute fold change above 1.2 were considered statistically significant. Gene ontology was performed using EnrichR[25]. Pathway and upstream regulator analysis was performed using Ingenuity Pathway Analysis (Qiagen, The Netherlands). Partial least squares discriminant analysis (PLS-DA) was performed using the *caret* and *pls* libraries[26]. PLS-DA model was validated using 5×5-fold repeated cross-validation. Model performance was evaluated using area under ROC curve (AUROC). Receiver Operator Characteristic (ROC) curve and heatmaps were made using *ROCR* and *ComplexHeatmap* libraries, respectively [27, 28].

Results

Vitamin D Status and Muscle Strength

Calcifediol supplementation led to significant increases in total 25(OH)D and 24,25(OH_2)D_3 levels compared to placebo (table 5.1). At the end of the study, subjects in the placebo group were on average still below the deficiency cut-off used for this study (50 nmol/L), whereas the calcifediol group was not. No differences were observed in muscle strength outcomes (BioDex leg extension and flexion peak torque, table 5.1). A full discussion of physiological outcomes of vitamin D_3 supplementation and calcifediol supplementation can be found elsewhere [16].

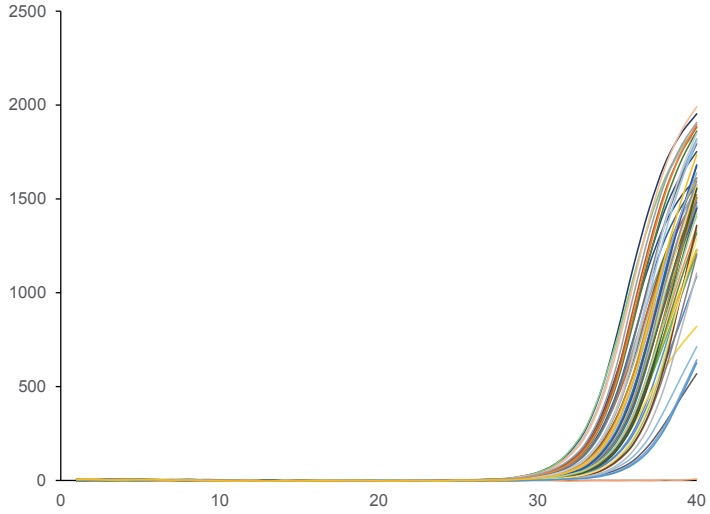
Table 5.1 – Subject characteristics and main effect of calcifediol supplementation

	Placebo - Pre	Placebo - Post	25(OH)D - Pre	25(OH)D - Post
N	12		10	
Gender (M / F)	6 / 6		6 / 4	
Age (years)	74.1 ± 5.8		71.8 ± 5.7	
BMI (kg/m²)	27.2 ± 4.0		28.0 ± 4.0	
Weight (kg)	76.2 ± 14.0		80.7 ± 14.9	
Body Fat (%)	32.1 ± 6.9	31.3 ± 6.6	32.5 ± 7.8	32.4 ± 7.8
Total 25(OH)D (nmol / L)	37.5 ± 11.9	43.8 ± 14.1	34.1 ± 9.3	87.3 ± 20.6 ***
24,25(OH_2)D_3 (nmol / L)	1.9 ± 0.8	2.7 ± 1.5	1.8 ± 0.7	8.0 ± 3.7 ***
BioDex Leg Extension Peak Torque (Nm)	129.7 ± 48.9	133.6 ± 64.1	145.9 ± 50.3	157.2 ± 56.1
BioDex Leg Flexion Peak Torque (Nm)	57.0 ± 29.9	55.2 ± 35.0	64.8 ± 22.5	70.0 ± 23.6

Effect of Calcifediol on Muscle Transcriptome

Overall, we were unable to find strong effects of calcifediol supplementation on the skeletal muscle transcriptome. Both the microarrays and qPCR revealed very low expression of the vitamin D receptor in our muscle biopsies (median raw intensity on microarrays of 12.1, amplification curves for the vitamin D receptor gene are shown in figure 5.1). Unadjusted P-values were uniformly distributed in both treatment groups and for the interaction between group and time (figure 5.2A,B,C). As a consequence, genes with a low p-value are likely to be false positives. Correcting for multiple testing using false discovery rate led to no differentially expressed genes using any sensible cut-offs (all q-values ~ 1). Using PLS-DA we attempted to separate the transcriptomic response to calcifediol supplementation from the response in the placebo group. This approach did not reveal any consistent patterns in the data (AUROC < 0.5 during 5×5-fold repeated cross-validation, figure 5.2D; accuracy of 0.46, Kappa of -0.12).

Figure 5.1 – qPCR Amplification curves of the vitamin D receptor in muscle biopsies



Using an optimistic p-value cut-off of 0.05 and signed fold change cut-off of |1.2| revealed 123 genes differentially regulated in the calcifediol group. The top 20 for genes changed in the calcifediol arm can be found in table 5.2. Significant genes did not include pathways or processes related to vitamin D receptor signalling (supplementary table 5.1 and 5.2). Genes previously described as putative target genes for the vitamin D receptor [29-31] did not show differential expression, with the exception of the insulin-like growth factor 1 receptor (IGF1R, p < 0.05, fold change of -1.27 for the interaction effect between time and treatment; figure 5.3).

Table 5.2 – Top 20 genes based on absolute fold change in the calcifediol arm

Gene	FC – 25(OH)D	FC - Pla.	P-val. - 25(OH)D	P-val. - Pla.	P-val. Interaction
SCD	2.01	1.19	0.002	0.374	0.069
S100A8	1.88	1.02	0.044	0.947	0.142
ANKRD1	1.80	1.52	0.020	0.065	0.600
HBB	1.64	1.01	0.039	0.976	0.125
LOC102724637	-1.62	-1.16	0.005	0.312	0.134
HBA2	1.57	-1.07	0.050	0.754	0.095
MIR4521	1.56	1.23	0.041	0.274	0.411
ALOX12-AS1	-1.55	-1.01	0.001	0.958	0.010
GTF2IP20	-1.52	-1.01	0.001	0.950	0.013
OXCT1	-1.45	-1.13	0.021	0.378	0.237
BORCS7	1.40	1.13	0.046	0.432	0.323
LOC101928316	-1.39	1.03	0.039	0.822	0.091
VSIG10	-1.39	-1.04	0.004	0.679	0.049
THRSP	1.39	-1.12	0.037	0.416	0.039
FMC1	-1.37	-1.01	0.012	0.959	0.060
MYLK3	-1.35	-1.09	0.014	0.431	0.172
CCDC80	1.35	1.20	0.028	0.139	0.495
LOC105378929	-1.34	-1.20	0.037	0.142	0.551
E2F6	-1.33	-1.16	0.014	0.147	0.359

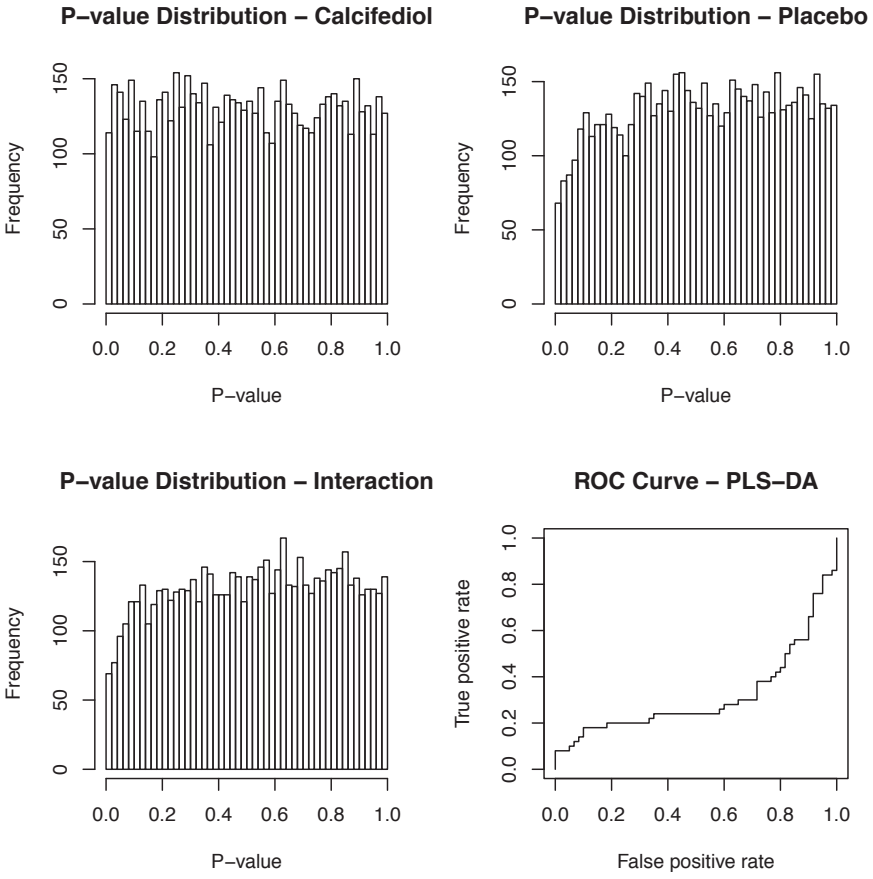


Figure 5.2 - A,B,C and D -Top left (A): P-values for the change in gene expression for all genes after filtering; before and after calcifediol supplementation. Uniform distribution (i.e. no increased frequency of genes for lower p-values) indicates an absence of an effect. Top right (B): values for the change in gene expression; before and after in the placebo group. Bottom left (C): P-values for the interaction effect (change in the calcifediol group vs. change in placebo group). Bottom right (D): receiver operator characteristic curve for the PLS-DA model.

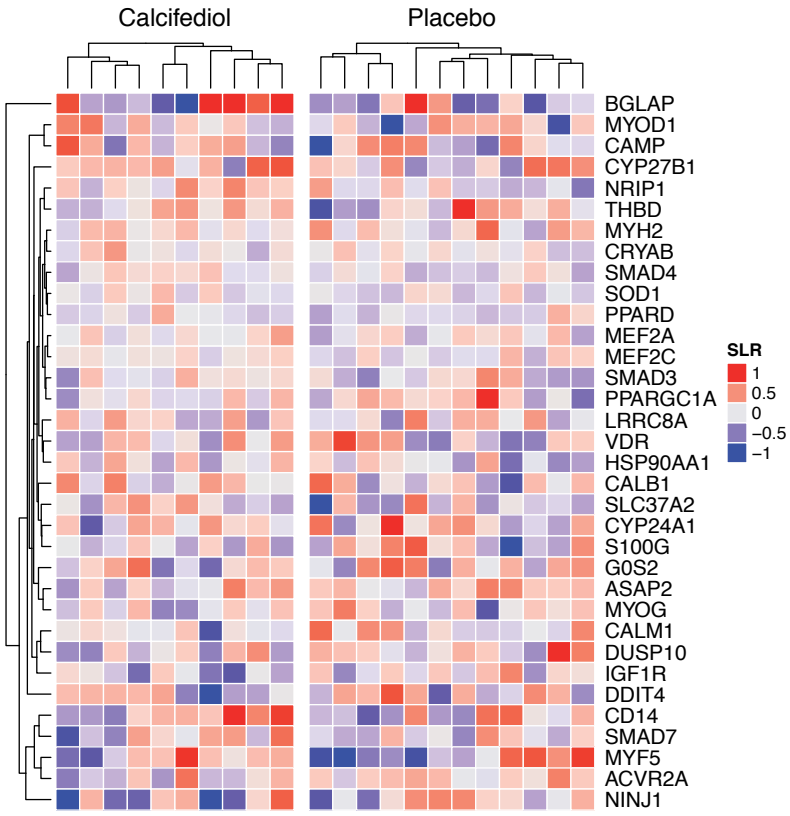


Figure 5.3 – Heatmap of gene expression changes (signal log ratio, SLR) of putative genomic targets of the vitamin D receptor

Discussion

Supplementation with calcifediol led to a significant increase in vitamin D status as determined by total 25(OH)D levels (34.1 ± 9.3 to 87.3 ± 20.6, $p < 0.001$). However, we were unable to confidently identify skeletal muscle genes that were affected by calcifediol supplementation in deficient pre-frail older adults. Neither a univariate technique (limma) nor a multivariate technique (PLS-DA) led to the identification of a robust signature of vitamin D supplementation in skeletal muscle. This is also in accordance with the lack of effect on muscle function [16].

Using somewhat permissive cut-offs we found 123 genes differentially regulated in the calcifediol supplemented arm. However, the same cut-offs led to 59 differentially regulated genes in the placebo group. Interaction contrast for time x treatment led to identification of 143 genes. Given the flat p-value distributions (figure 5.2), it is difficult to

attribute these differentially expressed genes to vitamin D supplementation without risking an unacceptable number of false positives.

Gene ontology analysis, pathway analysis and upstream regulator analysis of these genes also did not reveal any effects on pathways related to muscle function, vitamin D receptor signalling, calcium metabolism or phosphate metabolism (supplementary table S5.1 and S5.2). Known target genes of the vitamin D receptor were also not significantly affected (figure 5.3). Many of these genes were identified in immune cells, particularly microarray studies using immune cells [30]. ChIP-seq data suggests that the target genes for the vitamin D receptor can vary greatly depending on the cell type [32]. To our knowledge, no ChIP-seq analysis has been carried out on skeletal muscle cells. Other genes were selected based on a recent paper of by Hassan-Smith and colleagues (2017), where muscle gene expression was correlated with circulating levels of $1,25(OH_2)D_3$ and 25(OH)D. Correlations were generally weak (~0.3 – 0.5), but considered statistically significant.

There are several possible explanations for a lack of effect in this study. Importantly, there is still some discussion as to whether the vitamin D receptor is expressed in skeletal muscle tissue. Our data showed that the vitamin D receptor is very weakly expressed in our samples (figure 5.1). Reports of vitamin D receptor expression in skeletal muscle go back several decades [33, 34]. However, more recently it was revealed that antibodies for the vitamin D receptor may not be sufficiently specific, leading to overestimation of vitamin D receptor protein levels in skeletal muscle [35]. Olsson and colleagues observed that while precursor cells and myotubes express the vitamin D receptor, mature skeletal muscle does not [14]. This could also partly explain why we observed no significant effects, as we used whole muscle tissue rather than specific sub-populations of cells. The signal from these specific cells would be diluted by the signal from the muscle tissue as a whole. Since satellite cells are generally quiescent, it may be possible that the effect of vitamin D can only be observed once proliferation and differentiation processes are triggered.

Another possibility is that the participants in this study were not sufficiently deficient to observe an effect. We did not include individuals with a vitamin D status below 20 nmol/L due to ethical concerns. It is conceivable that very severe deficiency has a much stronger impact on muscle function, thus leading to a bigger observable effect. However, severe deficiency might also lead to alterations in calcium and phosphate metabolism, each of which can have an effect on muscle function [36, 37].

We suspected that deficient frail older adults were the most likely to benefit from vitamin D supplementation in relation to muscle function. Despite this, it seems that the effect of vitamin D on the muscle transcriptome is weak, if there even is an effect. Since it is possible that the vitamin D receptor is only present in proliferating satellite cells, it might be particularly difficult to observe a transcriptomic effect in whole skeletal muscle *in vivo*. Studies in subjects with more severe deficiency could potentially reveal more robust effects, but would likely also lead to the involvement of phosphate and calcium metabolism. In

short, our data suggest that vitamin D does not plays a significant role in gene regulation in human skeletal muscle.

Conclusion

We did not observe a robust effect of calcifediol supplementation on the skeletal muscle transcriptome among frail older adults in this randomized, double blind placebo-controlled clinical trial. Our findings indicate the effects of vitamin D supplementation on skeletal muscle transcriptome may be either absent, weak, or limited to a subset of muscle cells.

Conflicts of Interest

The project is funded by TI Food and Nutrition, a public-private partnership on precompetitive research in food and nutrition. The public partners are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The private partners have contributed to the project through regular discussion. LdG declares to have filed a patent related to vitamin D and cognitive executive function. All other authors have nothing to declare.

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Chapter 6 - The transcriptomic signature of muscle atrophy after knee immobilisation most closely resembles the transcriptomic signatures of frailty and age

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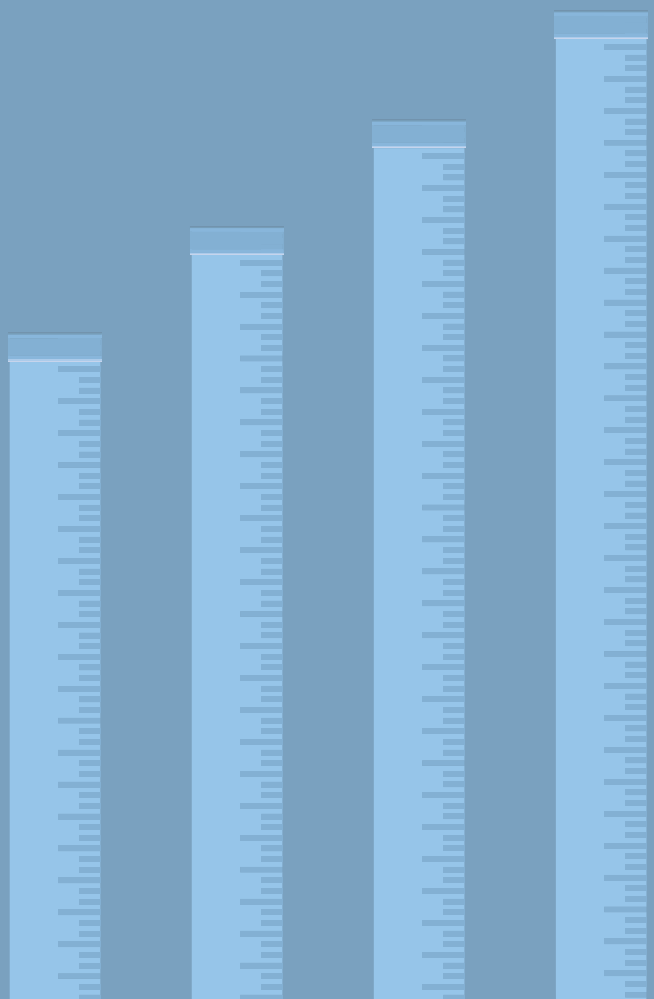

Abstract

Physical inactivity can lead to rapid loss of skeletal muscle mass and strength and negatively impacts overall health. Here we compare the transcriptomic changes between a week of bed rest and a week of knee immobilization, in addition to other available muscle atrophy datasets. Our goal was to determine the similarities and differences among various causes of muscle atrophy in humans. In addition, we aimed to assess which model for human disuse atrophy most closely resembles the transcriptomic signature of older age and age related frailty.

Muscle biopsies were obtained before and after 7 days of either bed rest or plaster cast knee immobilization. Gene expression microarrays were used to measure transcriptomic changes induced by muscle disuse. Additional data were obtained from the Gene Expression Omnibus (GEO) database and prior studies on the effect of knee immobilization, unilateral lower limb suspension, protein deficiency, bed rest, age and frailty on muscle gene expression.

Both knee immobilization and bed rest led to significant changes in the muscle transcriptome (861 and 708 differentially expressed genes, respectively). However, the overlap in significantly changed genes was relatively small (61 genes). Knee immobilization was characterized by ubiquitin-mediated proteolysis and induction of the HDAC4/Myogenin axis, whereas bed rest revealed increased involvement of the immune system and increased expression of lysosomal genes. Both atrophy models showed decreased expression of energy metabolism genes (glucose metabolism and oxidative phosphorylation). Systematic analysis of other studies revealed similar negative regulation of energy metabolism genes. We built regression models to estimate recent losses in muscle cross-sectional area (r2 of 0.35). Comparison of expression profiles revealed that knee immobilization was the closest to age and frailty signatures. Finally, canonical markers of muscle atrophy (FBXO32 and TRIM63) lacked robustness for the assessment of human muscle atrophy.

We showed a proof-of-concept technique of determining muscle atrophy using microarray data. Knee immobilization showed the highest similarity with age-related transcriptomic changes. This finding suggests that knee immobilization may be the most similar form of disuse atrophy to assess the effectiveness of strategies to prevent age-related muscle loss in humans. Our study suggests that the mechanisms of muscle atrophy vary across disuse models.

Introduction

Brief periods of muscle disuse can have lasting negative effects on muscle health and overall health. Not only does muscle disuse lead to rapid loss of muscle mass and strength, physical inactivity can also lead to a reduction in insulin sensitivity, increased adiposity and an overall decrease in metabolic rate [1-3]. Furthermore, it has been suggested that brief periods of muscle disuse accumulate over a lifetime, which might contribute to sarcopenia [4].

Muscle atrophy results from an imbalance between overall muscle protein synthesis and muscle protein breakdown rates. Which part of the equation is dominant during muscle loss (i.e. decreased synthesis or increased breakdown) is subject to discussion [5]. In addition, the underlying mechanisms of muscle atrophy can vary depending on the cause of the atrophy, making the study of muscle atrophy particularly difficult. Potential mechanisms for muscle protein breakdown include autophagy, apoptosis, calpains and ubiquitin-mediated proteolysis [6, 7].

Up until now the most frequently used method of presence of muscle atrophy at the molecular level, particularly over longer periods such as in relation to sarcopenia and cachexia, has been to measure expression of genes that are thought to mediate proteolysis in skeletal muscle. The most characterized genes in this context are FBXO32 and TRIM63, two ubiquitin E3 ligases thought to specifically mediate muscle protein breakdown, which were identified using transcriptomics studies in animal models [8-10]. These genes are believed to be common to many forms of muscle loss [11]. However, these genes have been inconsistent markers for muscle atrophy, particularly in human studies, and have been subject to some criticism [12, 13].

As a consequence, there is a need for a more comprehensive strategy to assess muscle atrophy at the molecular level, particularly in humans. Muscle disuse can be used to induce transient muscle atrophy in order to study underlying mechanisms in healthy humans *in vivo*. However, to complicate matters, there are various models for disuse atrophy, including bed rest, leg cast immobilization, and limb suspension. These models differ in various ways. For example, bed rest can affect muscles across the whole body, potentially leading to atrophy and metabolic effects in all muscles, whereas cast immobilization and limb suspension are limited to muscles in specific limbs. Bed rest can also potentially lead to other systemic changes, including increased inflammation and changes in levels of (stress) hormones [14]. Knee immobilization using a plaster cast, particularly when placed at an angle, prevents complete use of a limb. Milder models, such as unilateral lower limb suspension or knee immobilization using a brace, potentially do not completely remove muscle loading [15].

Many processes are involved in muscle atrophy. It can be hypothesized that that some of these processes are distinct for different causes of atrophy. Transcriptomics can be used to evaluate a wide range of underlying mechanisms simultaneously and could potentially shed light on how to more reliably study muscle atrophy in humans. Here we

compare the transcriptomic response to seven days of bed rest and seven days of knee immobilization in young healthy men. We also compare these responses to other publicly available datasets on muscle atrophy. Lastly, we aim to determine which source of muscle atrophy approximates the patterns in gene expression associated with decreased muscle mass and strength observed in frail older adults.

Materials and Methods

Study designs

Samples were derived from two studies performed previously. Both studies involved seven days of physical inactivity: either strict bed rest or full leg immobilization using a plaster cast. Participants were young, healthy men with no fractures or illnesses (subject characteristics can be found in table 1). Subjects who underwent leg immobilization were part of a randomized parallel-arm placebo controlled study on the effect of creatine supplementation during knee immobilization [16, 17]. Subjects who underwent bed rest were part of a study to assess the impact of bed rest on insulin sensitivity [18]. All samples were taken in an overnight fasted state. Muscle cross-sectional area (CSA) was determined using single slice CT scans. Strength loss was assessed using 1-repetition maximum (1RM) leg extension tests. These 1RM tests were unilateral for the knee immobilization study and bilateral for the bed rest study. On the test day before seven days of physical inactivity, the precise scanning position was marked with semi-permanent ink for replication on test day after the seven-day period of inactivity. More details on the experimental procedures and outcomes of these studies can be found in their respective papers [16, 18].

Microarray analysis

RNA was isolated (RNeasy Micro kit, Qiagen, Venlo, the Netherlands), quantified (Nanodrop ND 1000, Nanodrop technologies, Wilmington, DE, USA) and integrity was checked by an Agilent 2100 Bioanalyser with RNA 6000 microchips (Agilent Technologies, South Queensferry, UK). Total RNA was labelled using the GeneChip® WT plus Reagent Kit and hybridized to GeneChip® Human Gene 1.1 ST Arrays (Affymetrix, Inc. Santa Clara, CA, USA). Sample labelling, hybridization to chips, and image scanning were performed according to the manufacturers' instructions. All samples for the 7-day bed rest study and 7-day knee immobilization were analysed in a single batch.

Data analysis

Physical characteristics (i.e. muscle size and strength) were evaluated using linear mixed models. Microarray data were assessed for quality using the MADMAX pipeline [19] and by visually inspecting the probe level residuals and NUSE plots. Data was normalized using Robust Multichip Average (RMA) [20]. Gene level summarization was performed using the

most recent Custom CDF from the Brainarray project [21]. Genes were filtered using UPC filtering with a 50% expression likelihood cut-off in at least 8 samples, the smallest subset of samples within our dataset [22]. Statistical analysis was performed using the *limma* R library [23]. All models were evaluated using a paired analysis (after intervention versus before intervention). P-values were calculated using Intensity Based Moderated t-tests (IBMT) [24].Gene set enrichment analysis (GSEA) was performed on pre-ranked lists, ranked by the t-values from *limma*. We used the most recent library of canonical pathways from MsigDb [25]. Data visualization was done using C*ytoscape*, *ggplot2* and *gplots* [26-28]. Upstream regulator analysis was done using Ingenuity Pathway Analysis. An unadjusted p-value of 0.01 was considered significant for gene level (univariate) analysis. A q-value of 0.10 was considered significant for the GSEA results.

Regression analysis

We used a sparse partial least squares (sPLS) regression model to determine the recent change in muscle CSA. Relative change in muscle cross-sectional area (i.e. percentage of total CSA lost) of the quadriceps was used as dependent variable. Leg extension was chosen because the biopsies were obtained from the *m. vastus lateralis*. Two models were made: one where all genes after UPC filtering were used as independent variables (naïve model) and one model where the independent variables were genes that were significantly changed during both bed rest and knee immobilization in the placebo arm (common genes model). Data from the samples taken after physical inactivity were used for the model. The goal was to determine a signature of recent muscle atrophy in these samples. Hyperparameter selection and validation was done using leave-one-out cross-validation. Fitting procedure was performed using the *caret* library [29].

Atrophy model comparison and similarity network

We conducted a search on the GEO database for available datasets to assess similarities in transcriptomic changes between different forms of muscle atrophy. We searched for studies with human muscle biopsies derived from healthy volunteers. Only studies using commercially available microarrays were considered. We included data from two ULLS studies (GSE21496 and GSE33886) [30, 31], a two-day knee immobilization study (GSE5110) [32], a bed rest study (GSE24215)[33] and a protein deficiency study (GSE8441)[34]. All but one study used Affymetrix chips and all studies were performed in healthy human volunteers. A single study used Agilent arrays. An overview of the studies we obtained from GEO can be found in table 6.1.

Table 6.1 – Description of datasets obtained from the GEO database.

GEO Accession	Model Type	Array Type	Duration	Study
GSE24215	Bed Rest	Agilent 4x44K G4112F	9 days	Alibegovic et al., 2010 [33]
GSE33886	Unilateral Lower Limb Suspension	Affymetrix HuGene 1.0 ST	21 days	Lammers et al., 2011 [31]
GSE21496	Unilateral Lower Limb Suspension	Affymetrix HGU133 Plus 2	1 day	Reich et al., 2010 [30]
GSE21496	Unilateral Lower Limb Suspension	Affymetrix HGU133 Plus 2	2 days	Reich et al., 2010 [30]
GSE8441	Protein Deficiency	Affymetrix HGU133A	7 days	Thalacker-Mercer et al., 2007 [34]
GSE5110	Knee Immobilization (brace)	Affymetrix HGU133 Plus 2	2 days	Urso et al., 2006 [32]

All Affymetrix datasets were summarized at the gene level using Brainarray custom CDFs and data was normalized using RMA. The Agilent dataset was downloaded pre-normalized from GEO and gene level expression was summarized using median expression per gene. Statistical testing was done by assessing changes in the transcriptome compared to baseline after physical inactivity or protein deficiency, as with the 7-day bed rest and knee immobilization studies. To approximate age-related muscle loss we used samples from a study we published previously on transcriptomic differences between young subjects, frail older subjects and healthy older subjects [35]. The effect of frailty and age on the transcriptome were analysed cross-sectionally, adjusted for sex.

To assess similarities between models we used the Bioconductor R library *OrderedList* [36]. This library assesses the similarities based on the order of ranked lists. In our case all comparisons were ordered based on the gene level t-test statistic, where each intervention was compared to all other comparisons in a pairwise fashion. We specified that 50% of the genes were invariant and we used unfiltered datasets for the comparisons. Some genes were dropped for some of these comparisons due to differences in chip architectures. The similarity scores and p-values were collated and were integrated and visualized using Cytoscape [28]. We made a force-directed graph based on the similarity score to visually represent similarities between interventions using distance. Some nodes were manually moved slightly to enhance legibility of the figure.

Results

Knee immobilization

The effect of knee immobilization on muscle size and strength was published previously [16]. Likewise, the effect of creatine supplementation on the muscle transcriptome during knee immobilization was also discussed elsewhere. Briefly, seven days of knee immobilization induced rapid loss of muscle mass. Leg CSA was reduced by 574 ± 115 mm^2 and quadriceps CSA was reduced by 484 ± 65 mm^2. These changes were accompanied by a reduction in unilateral leg extension 1RM of 7.4 ± 1.8 kg. Creatine supplementation did not have a significant effect on the loss of both muscle mass and muscle strength (table 4.1). However, we did observe that generally gene expression changes were less pronounced in the creatine group. A full discussion of the effects of creatine supplementation during leg immobilization on the muscle transcriptome and metabolome can be found in chapter 4 [17].

Table 6.2 – descriptive statistics of study population. Asterisks indicate significant difference compared to baseline. * = p < 0.05, ** = p < 0.01, *** p < 0.001

	KI		Bed Rest	
	Pre	Post	Pre	Post
N	8		9	
Age (years)	22.9 ± 3.2		23.0 ± 2.1	
height (m)	1.75 ± 0.09		1.81 ± 0.08	
Weight (kg)	71.4 ± 14.5		76.4 ± 6	
BMI (kg/m²)	23.1 ± 3.3		23.5 ± 2.4	
Bilateral Leg Extension 1RM (kg)			131.1 ± 19.8	120.6 ± 19.8*
Lean Body Mass (kg)			56.9 ± 3.8	55.9 ± 4.0**
Body Fat (%)			21.2 ± 6.8	21.9 ± 7.2
Unilateral Leg Extension 1RM (kg)	53.3 ± 15.3	52.4 ± 16.5		
Whole Leg CSA (mm²)	14510 ± 2004	14004 ± 1981**	14430 ± 1610	14151 ± 1728
Quadriceps CSA (mm²)	7844 ± 1363	7428 ± 1313***	8060 ± 911	7843 ± 1024*

Among the top 20 significant genes for knee immobilization were genes related to energy metabolism, as well as genes related to neuromuscular function (table 6.3, discussed in chapter 4). GSEA revealed increased expression of proteasomal genes and ubiquitin mediated proteolysis pathways, whereas glycolysis, gluconeogenesis and mitochondrial function pathways were downregulated (supplementary table S6.1). Upstream regulators analysis showed decreased expression of insulin target genes and increased expression of Forkhead box O3 (FOXO3), which was consistent with the downregulation of glucose metabolism genes and upregulation of proteolytic genes in the GSEA results (supplementary table S6.2).

Table 6.3 – Top 20 genes for knee immobilization, bed rest and genes significant in both disuse atrophy models. Genes were ranked by absolute fold changes after selecting for significance ($p < 0.01$). Rank product was used on the absolute fold changes to rank the genes for the common genes. FC is signed fold change, KI is knee immobilization and BR is bed rest.

Gene	Description	FC BR	FC KI	P-val. BR	P-val. KI
Knee Immobilization					
NMRK2	*nicotinamide riboside kinase 2*	-1.76	-5.30	0.027	0.000
GDNF	*glial cell derived neurotrophic factor*	-1.17	4.80	0.521	0.000
SMCO1	*single-pass membrane protein with coiled-coil domains 1*	-2.92	-4.74	0.008	0.001
ACTN3	*actinin alpha 3*	1.11	-4.43	0.735	0.000
NR4A3	*nuclear receptor subfamily 4 group A member 3*	-1.90	-4.05	0.100	0.002
NR4A1	*nuclear receptor subfamily 4 group A member 1*	-1.73	-3.72	0.037	0.000
IL18	*interleukin 18*	2.06	3.51	0.064	0.004
HDAC4	*histone deacetylase 4*	-1.02	3.20	0.888	0.000
DHRS7C	*dehydrogenase/reductase 7C*	-1.70	-3.15	0.097	0.002
PDE11A	*phosphodiesterase 11A*	1.52	2.91	0.045	0.000
GADL1	*glutamate decarboxylase like 1*	-2.30	-2.88	0.027	0.009
AMPD3	*adenosine monophosphate deaminase 3*	1.42	2.82	0.224	0.002
CA14	*carbonic anhydrase 14*	-2.60	-2.82	0.011	0.010
GADD45A	*growth arrest and DNA damage inducible alpha*	2.34	2.69	0.005	0.002
SLC25A30	*solute carrier family 25 member 30*	-1.89	-2.66	0.029	0.003
LRRC14B	*leucine rich repeat containing 14B*	-1.65	-2.59	0.048	0.001
TP63	*tumor protein p63*	1.32	2.58	0.061	0.000
AKR1C2	*aldo-keto reductase family 1 member C2*	1.00	2.56	0.994	0.000
SLC16A3	*solute carrier family 16 member 3*	-1.36	-2.54	0.051	0.000
COQ10A	*coenzyme Q10A*	-1.91	-2.48	0.006	0.001
Bed Rest					
MYH8	*myosin heavy chain 8*	14.81	2.00	0.001	0.361
MYH3	*myosin heavy chain 3*	13.71	1.36	0.001	0.668
TNNT2	*troponin T2, cardiac type*	12.75	1.05	0.000	0.945
SPP1	*secreted phosphoprotein 1*	10.40	-3.30	0.006	0.165
CHRNA1	*cholinergic receptor nicotinic alpha 1 subunit*	8.83	3.09	0.000	0.026
MYBPH	*myosin binding protein H*	8.46	2.66	0.001	0.126
TMEM8C	*transmembrane protein 8C*	7.03	1.36	0.001	0.571
MYL4	*myosin light chain 4*	6.11	1.41	0.000	0.462
DCLK1	*doublecortin like kinase 1*	5.84	1.04	0.001	0.935
LRRC17	*leucine rich repeat containing 17*	5.47	-1.03	0.002	0.959
LAPTM5	*lysosomal protein transmembrane 5*	5.32	-2.36	0.006	0.153
CHI3L1	*chitinase 3 like 1*	5.19	1.19	0.007	0.778
PRG4	*proteoglycan 4*	5.14	-2.48	0.005	0.117

PRUNE2	*prune homolog 2*	5.10	2.24	0.005	0.160
IFITM10	*interferon induced transmembrane protein 10*	4.65	1.07	0.002	0.886
CD68	*CD68 molecule*	4.62	-2.11	0.004	0.157
FPR3	*formyl peptide receptor 3*	4.51	-2.35	0.003	0.093
LYZ	*lysozyme*	4.31	-2.07	0.009	0.194
MSR1	*macrophage scavenger receptor 1*	4.28	-1.64	0.003	0.301
C1QB	*complement C1q B chain*	3.96	-1.91	0.003	0.158
Common Genes					
SMCO1	*single-pass membrane protein with coiled-coil domains 1*	-2.92	-4.74	0.008	0.001
GADD45A	*growth arrest and DNA damage inducible alpha*	2.34	2.69	0.005	0.002
CCDC39	*coiled-coil domain containing 39*	3.57	2.17	0.000	0.009
COQ10A	*coenzyme Q10A*	-1.91	-2.48	0.006	0.001
MYOG	*myogenin*	2.13	2.01	0.001	0.003
GLRX	*glutaredoxin*	1.95	2.02	0.001	0.001
ARPP21	*cAMP regulated phosphoprotein 21*	1.62	2.28	0.002	0.000
CDC42EP3	*CDC42 effector protein 3*	1.53	2.26	0.009	0.000
MPP6	*membrane palmitoylated protein 6*	1.49	2.25	0.002	0.000
MEGF10	*multiple EGF like domains 10*	1.86	1.79	0.001	0.002
YPEL1	*yippee like 1*	1.36	1.97	0.004	0.000
GOT1	*glutamic-oxaloacetic transaminase 1*	-1.68	-1.63	0.003	0.006
MYOZ3	*myozenin 3*	-1.73	-1.58	0.001	0.008
AMOT	*angiomotin*	-1.56	-1.65	0.009	0.006
CALM1	*calmodulin 1*	1.40	1.75	0.001	0.000
SLC1A4	*solute carrier family 1 member 4*	-1.40	-1.73	0.002	0.000
SLC29A1	*solute carrier family 29 member 1*	-1.42	-1.70	0.007	0.000
RIMKLB	*ribosomal modification protein rimK like family member B*	1.48	1.61	0.001	0.000
RASGRP3	*RAS guanyl releasing protein 3*	-1.45	-1.63	0.009	0.002
TP53INP1	*tumor protein p53 inducible nuclear protein 1*	1.58	1.46	0.000	0.003

Bed rest

Like knee immobilization, bed rest led to rapid loss in muscle mass and strength. Lean body mass, as measured using DXA, was decreased by 1.4 ± 0.3 kg ($p < 0.01$) and leg CSA and quadriceps CSA were reduced by 279 ± 140 mm^2 (not significant) and 216 ± 67 mm^2 ($p < 0.05$), respectively. Leg extension 1RM was reduced by 5.0 ± 1.7 kg ($p < 0.05$) following bed rest. A full discussion of the effect on muscle morphology and insulin sensitivity can be found elsewhere [18].

Among the top 20 genes (table 6.3) that were upregulated by bed rest are genes related to muscle development, including developmental myosin heavy chain isoforms (MYH3, embryonic and MYH8, perinatal), troponin T2 (TNNT2), myosin binding protein H

(MYBPH), Transmembrane Protein 8C (TMEM8C) and myosin light chain 4 and 5 (MYL4 and MYL5). A marker of macrophages, CD68, was also among the top 20 genes. Further down the list are other genes expressed by macrophages and other immune cells, including CD163, CD14, CD4 and CD37 and various complement system genes, all showed increased expression compared to baseline.

Pathway analysis using GSEA showed decreased expression of genes involved in oxidative phosphorylation, glucose metabolism and fat metabolism. Upregulated pathways included lysosomal, apoptosis and various immune pathways, as well as tissue remodelling and angiogenesis pathways (supplementary table S6.3). Gene sets associated with ubiquitin mediated proteolysis were not significantly affected by bed rest using our cut-off ($p < 0.01$) Upstream analysis using ingenuity revealed inhibition of several energy metabolism regulators including PPARα, PPARδ and PGC1α. Other regulators that were affected included KDM5A (activated), KLF15 (inhibited), mTOR (affected), IGF1R (inhibited) and insulin (affected). The full results of the upstream analysis for the effect of bed rest on the transcriptome can be found in the supplementary materials (supplementary table S6.4).

Model comparisons

Seven days bed rest or knee immobilization

Bed rest and knee immobilization showed distinct effects on the transcriptome. Bed rest caused 707 genes differentially expressed genes, whereas knee immobilization led to 861 differentially expressed genes. Only 61 genes were differentially expressed in both disuse atrophy models. Of those 61 genes, 8 genes showed opposite regulation. A scatterplot comparing the change in expression (signal log ratios) of both interventions can be found in figure 6.1, highlighting the differences in transcriptomic responses. Genes significantly changed in both interventions included genes related to muscle development and muscle atrophy such as Myogenin, Multiple EGF Like Domains 10 (MEGF10) and growth arrest and DNA damage inducible alpha (GADD45A). Significant overlapping genes also included protein ubiquitination genes: UBE2E1, ZNF168 and UBE2H.

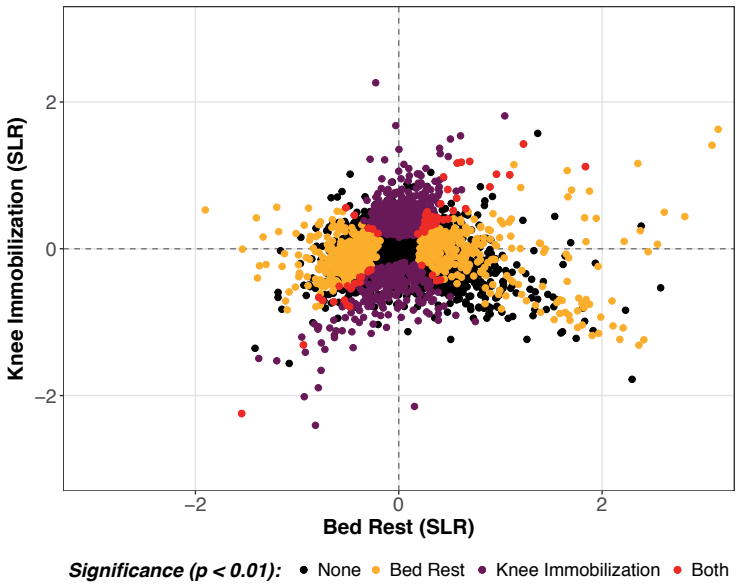

Figure 6.1 - Signal log ratios of gene expression changes during 7 days of knee immobilization versus 7 days of strict bed rest

GSEA and upstream analysis revealed that both forms of disuse atrophy led to decreased expression of mitochondrial function genes and energy metabolism genes. Comparison of the results suggests that bed rest seemed to have a larger impact on lipid metabolism, whereas knee immobilization seemed to have a bigger impact on glucose metabolism genes (supplementary tables S6.1 and S6.3). There were also dissimilarities between potential mechanisms underlying muscle atrophy: knee immobilization showed significant increases in ubiquitin mediated proteolysis genes and proteasomal genes, while bed rest suggests involvement of the immune system, as shown by increased expression of immune, apoptosis and lysosomal genes. Both models showed differential expression of genes related to neurological function, including axon guidance genes and acetylcholine receptor signalling (table S6.3).

Other muscle atrophy models

We compared six other microarray datasets investigating muscle atrophy. These varied in platform, duration and atrophy model. An overview of included datasets can be found in table 6.1. Only a single gene was significant in 7 of the 8 comparisons: Cholinergic Receptor Nicotinic Alpha 1 Subunit (CHRNA1). This gene was not significant in the seven-days knee immobilization using our cut-offs (p-value < 0.01), but it approached statistical significance (p-value 0.026). Collagen 4A1 was significant in 6 of the 8 comparisons, and 6 genes were significant in 5 of the 8 comparisons, including MEGF10 and calmodulin (CALM1) which were also among the common

genes between 7 days of bed rest and knee immobilization. Other genes that were significant in several models include collagens, and genes involved in oxidative phosphorylation and energy metabolism. A heatmap of the top 50 genes across these six datasets can be found in figure 6.2.

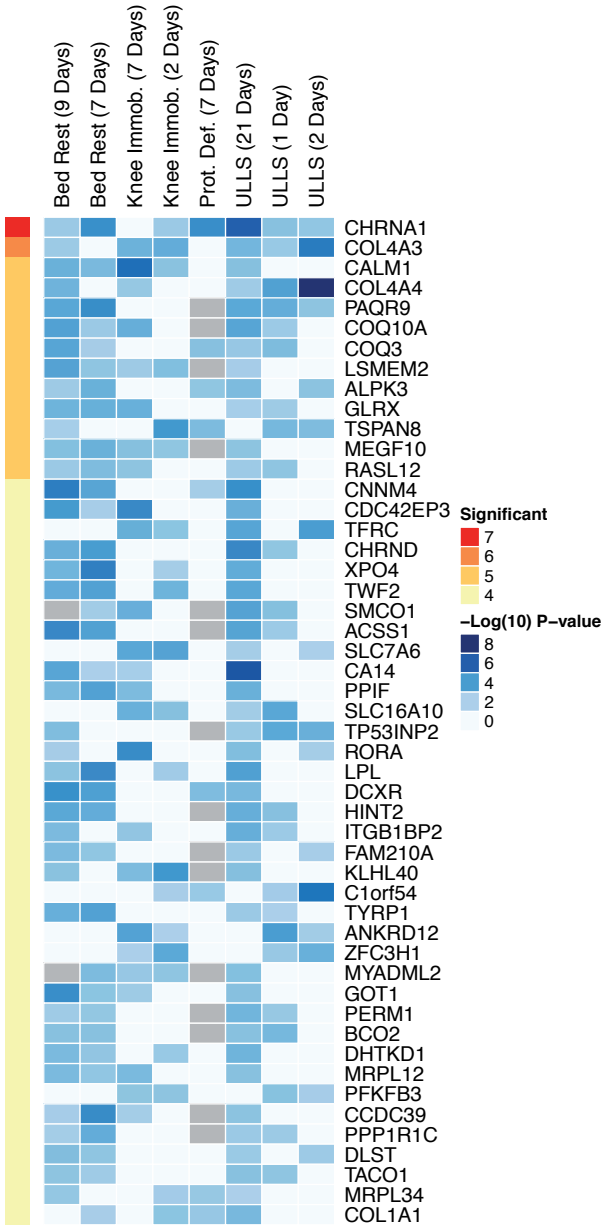

Figure 6.2 – Top 50 genes across muscle atrophy datasets based on rank product. Color intensity denotes $-\log_{10}$ of the p-value. Coloured bar on the left shows the amount of genes significant at $p < 0.01$. Genes with a higher p-value (i.e. $-\log_{10}$ of 2) were set to 0 to improve legibility. Grey values indicate that the gene was missing on the array.

As with 7 days of bed rest and knee immobilization, significant pathways included oxidative phosphorylation, TCA cycle and energy metabolism pathways. Upstream regulator analysis also showed downregulation of regulators involved in energy metabolism (peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC1α; peroxisome proliferator activated receptors alpha, gamma and delta, PPARα, PPARγ and PPARδ) and anabolic signalling (insulin receptor, INSR; insulin like growth factor 1 receptor; IGF1R). An overview of upstream regulators that appear common to all models can be found table 6.4.

Table 6.4 – Upstream analysis of genes common to all atrophy models. P-values were derived using Fisher's method across all datasets. Signal log ratios were calculated by taking the mean signal log ratio across interventions.

Upstream Regulator	Predicted Activation State	Activation z-score	p-value of overlap
INSR	Inhibited	-5.699	0.000
KDM5A	Activated	5.312	0.000
MAP4K4	Activated	5.555	0.000
PPARGC1A	Inhibited	-5.793	0.000
ESRRA	Inhibited	-4.347	0.000
Esrra	Inhibited	-3.85	0.000
RICTOR	Activated	6.297	0.000
RB1	Inhibited	-3.782	0.000
LONP1	Activated	2.784	0.000
OGT		0.64	0.000
MED30			0.000
INS		1.101	0.000
PPARA	Inhibited	-4.28	0.000
IGF1R	Inhibited	-3.848	0.000
Insulin		0.364	0.000
APP		0.648	0.000
VEGFA		-1.942	0.000
FOXO1		-0.725	0.000
PPARG	Inhibited	-3.83	0.000
SURF1	Activated	2.345	0.000

Similarity network

We made a network of all the included studies to get an overview of similarities between the various atrophy models (figure 6.3). We also included data on the effect of frailty and age on the skeletal muscle transcriptome to determine which atrophy model is most similar to the effect of frailty and age. This analysis revealed that the different modes of inducing disuse atrophy were similar, despite differences in duration. The two bed rest studies showed a transcriptional pattern that tended to be dissimilar from the full knee immobilization interventions but not ULLS. Protein deficiency showed the least overlap with other studies, but did show strong similarities with the bed rest and 3 weeks of ULLS.

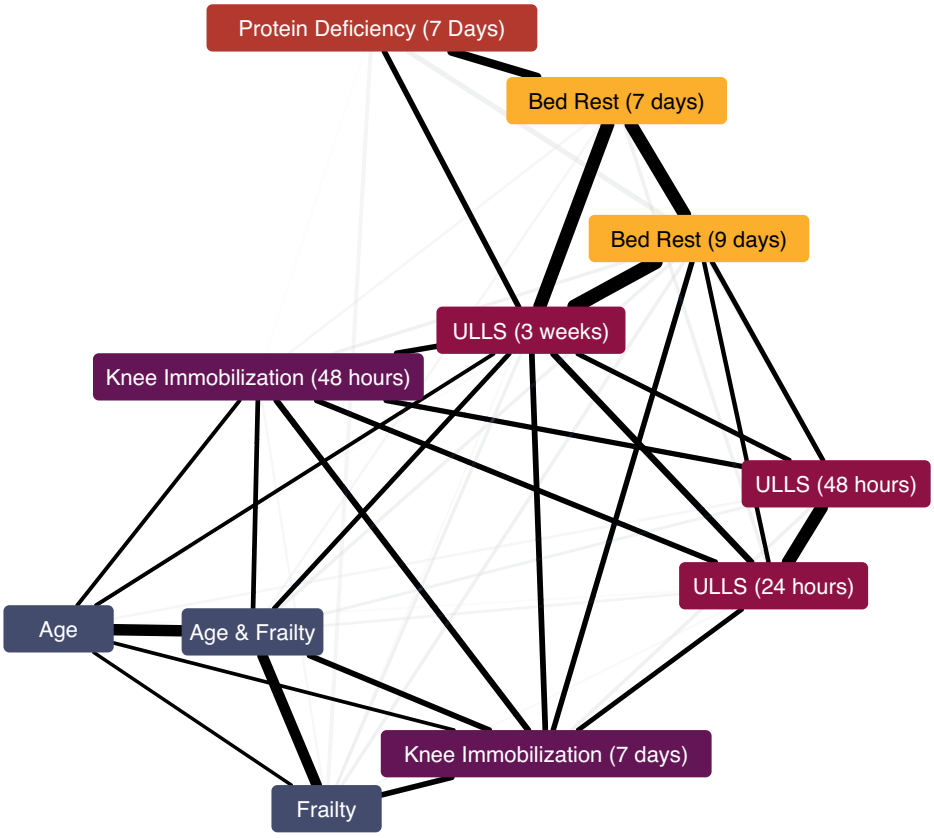

Figure 6.3 - similarity network for muscle atrophy models in humans. Black lines indicate significant similarity ($p < 0.01$). Line width is proportional to similarity score (wider lines indicate higher similarity).

Muscle Atrophy Prediction

We made two sPLS regression models (figure 6.4) to predict the amount of muscle loss that has occurred in the past 7 days in the participants: a naïve model with all genes after filtering as input and a model that used the genes significantly changed after both bed rest and knee immobilization. The final naïve model after grid search included 258 genes after sPLS feature selection, based upon two components with a sparsity parameter (eta) of 0.7. Cross-validation of the naïve model showed an r^2 of 0.25 and RMSE of 1.95 on the held-out samples during cross-validation. Predictive genes were primarily genes involved in ubiquitin mediated proteolysis after EnrichR analysis of selected predictor genes (table 6.3). The final model using only significant genes as predictors selected 27 genes, had 5 components and sparsity parameter of 0.7. Cross-validation showed an r^2 of 0.35 and RMSE of 1.78. Variable importance of predictor genes for the common genes model is shown in supplementary figure S6.1.

Table 6.5 – Top 10 enriched Gene Ontologies among predictive genes from the naïve sPLS regression model (GO biological processes).

Term	GO Accession	P-value	Adjusted P-value
modification-dependent macromolecule catabolic process	GO:0043632	0.000	0.003
modification-dependent protein catabolic process	GO:0019941	0.000	0.005
proteolysis involved in cellular protein catabolic process	GO:0051603	0.000	0.009
ubiquitin-dependent protein catabolic process	GO:0006511	0.000	0.023
protein catabolic process	GO:0030163	0.000	0.023
proteasome-mediated ubiquitin-dependent protein catabolic process	GO:0043161	0.000	0.104
proteasomal protein catabolic process	GO:0010498	0.000	0.117
mRNA processing	GO:0006397	0.001	0.146
RNA splicing	GO:0008380	0.001	0.146
regulation of proteasomal ubiquitin-dependent protein catabolic process	GO:0032434	0.001	0.146

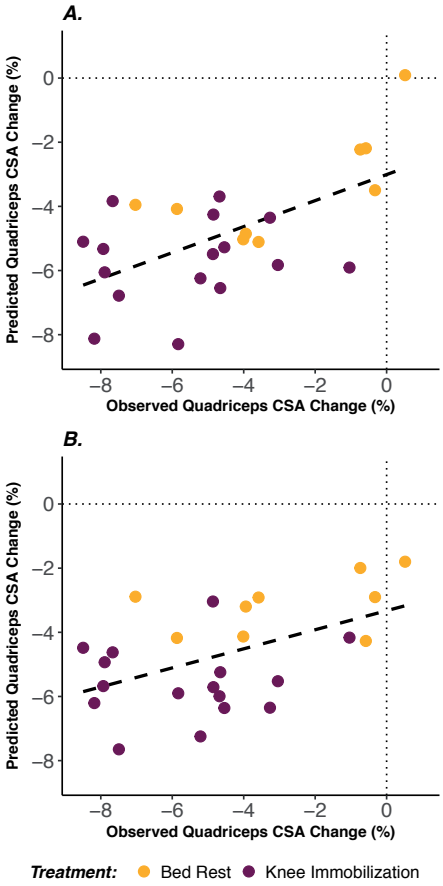

Figure 6.4 - Predicted versus observed muscle atrophy from sPLS regression models. Data points are from the held-out samples during leave-one-out cross-validation. 3A is for the sPLS model using only the genes significant in both 7 days of bed rest and 7 days of knee immobilization (61 genes; common genes model). 3B is for the naïve model using all genes after UPC filtering as input.

Discussion

Disuse atrophy can lead to rapid losses of muscle mass and muscle strength, as previously shown in a wide array of studies [37]. Here we compare two models for disuse atrophy: strict bed rest and knee immobilization using a leg cast. Bed rest and knee immobilization led to comparable losses in muscle mass and strength in the leg (table 6.1, [14]). However, we observed distinct transcriptomic responses to either 7 days of bed rest or 7 days of knee immobilization. Both studies were carried out at Maastricht University, had a similar population (young healthy men, table 6.1) and had the same duration of disuse (7 days). There are of course obvious differences between bed rest and knee immobilization as

models for disuse atrophy: bed rest-induced disuse atrophy leads to muscle losses over the whole body, whereas knee immobilization has localized effects. Our data suggest that there are major differences on a transcriptomic level in the vastus lateralis.

Predominant changes during knee immobilization are downregulation of energy metabolism genes and upregulation of ubiquitin mediated proteolysis genes. The latter consisted of both ubiquitination genes and proteasomal genes. Top genes for knee immobilization included HDAC4, a gene that is primarily associated with denervation induced muscle atrophy as part of the HDAC4/Myogenin axis [38]. Also among the top genes are various neuronal genes, suggesting a compensatory response to enhance neuronal survival and signalling via induction of glial derived neurotrophic factor (GDNF), increased expression of acetylcholine receptor subunits and decreased expression of acetylcholine esterase (ACHE) (table 6.3). A more detailed discussion of the effect of knee immobilization on the transcriptome and metabolome can be found in chapter 4 [17].

Bed rest had distinct effects on the muscle transcriptome compared to knee immobilization. Among the top 20 are several genes that are associated with muscle regeneration, such as embryonal and perinatal myosin heavy chain isoforms MYH3 and MYH8 and TMEM8C. There were also various genes involved in muscle contraction, cytoskeleton organisation and extracellular matrix genes (primarily collagen genes). In aggregate these gene expression changes suggest significant tissue remodelling and regeneration. There were signs of involvement of the immune system, as markers of immune cells were strongly upregulated (table 6.3), particularly macrophage markers and phagosomal genes. Among the top upregulated genesets were lysosomal genes, whereas ubiquitin mediated proteolysis genes were not significantly upregulated. These differences suggest that muscle atrophy during bed rest is mediated via a different mechanism than knee immobilization, most likely involving autophagy. This pattern, i.e. the transcriptional response to bed rest being distinct from knee immobilization, is also evident on a global scale of gene expression, as shown in figure 6.1 and 6.3. Previously, bed rest has been associated with modulation of stress hormones and immune markers, which may also have had an effect on insulin sensitivity and anabolic resistance [18, 39, 40].

We analysed one other study where bed rest was used as a model for muscle atrophy with a similar duration (9 days versus 7 days). However, some methodological differences exist between the studies: our study did not allow any visits to the bathroom, different microarray platforms were used (Agilent 4x44K G4112F vs. Affymetrix HuGene 2.1 ST), and subjects were in an insulin stimulated state when muscle biopsies were collected in the study by Alibegovic *et.al*. Despite these methodological differences we observed very similar effects on the muscle transcriptome: decreased energy metabolism genes and increased expression of tissue remodelling genes and inflammation genes. Similar effects on upstream regulators were also observed, with inhibition of PGC1A and several PPARs (table 6.4). The two studies showed a statistically significant similarity in our similarity network. However, our study showed less similarity with the unilateral lower limb suspension studies,

possibly because the bed rest was stricter in our study as compared to Alibegovic *et al.*. We also did not observe ER stress-related effects in our bed rest study, though genes related to protein synthesis and processing were upregulated in our study.

While plenty of genes were changed during both knee immobilization (861 genes) and bed rest (708 genes), the overlap in significantly affected genes was relatively small (61 genes). The overlapping genes include several genes that are associated with muscle function or atrophy such as MEGF10 and GADD45A. GADD45A is suspected to be a key regulator of muscle atrophy, and was upregulated in both bed rest and knee immobilization (table 6.3) [41, 42]. MEGF10 is associated with myogenesis and mutations in this genes are linked to myopathy [43]. A gene that is less obviously related to muscle function is the top gene SMCO1. Both bed rest and knee immobilization led to significant downregulation of SMCO1. The function of SMCO1 is currently unknown, but it is highly expressed in muscle tissue. Deletion of the 3q29 region containing this gene may be associated with neurological and developmental problems. Curiously, Ring Finger Protein 168, a gene that is also significantly upregulated during bed rest and knee immobilization, is hypothesized to bind this genomic region [44]. The only other literature reference on SMCO1 that we could find is an abstract, which describes SMCO1 as a muscle-specific gene and possibly regulated via Myocyte Enhancement Factor 2 (MEF2) [45].

Similarities with other models

When evaluating all studies on skeletal muscle atrophy in humans that used transcriptomics we did not find any genes that are significantly regulated among all studies. This could partly be explained by the heterogeneity in methodology, atrophy models and study duration. However, the comparison of all these studies did reveal some interesting candidates that seem relatively robust among all interventions. For example, MEGF10 and SMCO1 and are among the top significant genes across all studies (figure 6.2, table 6.3, supplementary table S6.5).

Common patterns across all studies include tissue remodelling (e.g. collagen and cytoskeleton reorganization genes), decreased energy metabolism (glucose metabolism, fat metabolism and oxidative phosphorylation) and neuromuscular changes (e.g. acetylcholine receptor subunit genes). Upstream regulator analysis also revealed that all models have a strong impact on energy metabolism, with inhibition of regulators of lipid metabolism, glucose metabolism, mitochondrial function genes and exercise response. A likely explanation of these changes is due to the lower energy demand of inactive skeletal muscle.

The canonical muscle atrophy genes FBXO32 and TRIM63 were not strongly regulated, with the largest significant fold change occurring for FBXO32 after 2 days of ULLS (FC = 1.51, p = 0.004). FBXO32 showed upregulation in the three studies with a shorter duration of disuse when using a p-value cut-off of 0.05 (1 and 2 days of ULLS and 2 days of knee immobilization). TRIM63 only showed significant differential expression after 1 day of ULLS. These data suggest that gene expression of these two genes are poor markers for more prolonged periods of muscle atrophy.

Similarity network

One of our goals for this study was to evaluate whether disuse atrophy models can be used as a surrogate for the study of age-related muscle mass loss and muscle weakness (i.e. sarcopenia and dynapenia). Sarcopenia occurs over a prolonged period of time, which makes it difficult to study. Furthermore, clinical sarcopenia is quite severe and it may be unethical to study strategies that might negatively affect muscle mass in this population. Ideally, if we are to evaluate strategies that can preserve muscle mass and function in atrophic situations, we would be able to use healthy young individuals that can bounce back relatively quickly from physical insults such as disuse-induced muscle atrophy. To address this question we made a similarity network.

The similarity analysis revealed that of all the models in this study, knee immobilization for 7 days showed the highest similarity with frailty (figure 6.3). Bed rest for 7 days and protein deficiency were the furthest removed from the effects of both age, frailty and a combination of age and frailty. This supports the notion that bed rest is primarily associated with a clinical outcome, whereas knee immobilization appears to be the most relevant for muscle atrophy itself. The unilateral lower limb suspension models appeared to be intermediates between bed rest and knee immobilization.

The high similarity between protein deficiency and bed rest is remarkable. Thalacker-Mercer *et al.* had previously observed very similar effects to our bed rest dataset and with the protein deficiency dataset, also observing involvement of the immune system and upregulation of lysosomal genes [34]. With regard to muscle atrophy, these results seem to suggest that autophagy and the immune system are primarily involved in muscle losses during bed rest and protein deficiency, whereas proteasomal systems (i.e. ubiquitin-mediated proteolysis and possibly apoptosis) are primarily involved in the age, frailty and knee immobilization side of the spectrum.

Muscle atrophy model

The models we built to detect recent muscle atrophy showed reasonable predictive capabilities, despite the relatively small sample size for such an approach. For the naïve model, selected genes were primarily genes involved in ubiquitin-mediated proteolysis, a process that is often the main mechanism for muscle atrophy (table 6.5). However, it is also very likely that both the type of disuse atrophy and the duration of disuse atrophy have an effect on which genes are involved in the loss of muscle mass. As a result, this model should be viewed as a proof-of-concept rather than a functional method of determining muscle loss. Larger sample sizes, different types of disuse atrophy, and multiple time points are needed to more accurately use gene expression microarrays as read-out for muscle atrophy. The second model using only genes significantly differentially expressed after both bed rest and knee immobilization highlighted YPEL1 as the most predictive of recent muscle loss.

Conclusions

Knee immobilization and bed rest have distinct effects on the human skeletal muscle transcriptome. Comparison of both forms of disuse atrophy revealed that bed rest was characterized by changes in expression of genes involved inlysosomal protein degradation and the immune system, whereas knee immobilization was characterized by proteasomal proteolysis. The effect of knee immobilization showed the highest similarity with the transcriptomic signature of age and frailty. Based on the transcriptome, strategies that prevent muscle atrophy during knee immobilization may have the highest predicted value to be beneficial in the prevention of age-related muscle loss.

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Chapter 7 – General discussion

Signatures of muscle status in the transcriptome

Chapter 2 describes changes in the transcriptome associated with age, frailty and resistance-type exercise training. We observed that age affects a wide range of genes and that frailty presents itself as a more pronounced effect of age. In the context of figure 1.1 in chapter 1, the frail and pre-frail individuals described in chapter 2 are further along the downward curve towards disability, as their phenotype classification based on the Fried criteria suggests [1]. Importantly, we also observe a shift towards a younger phenotype upon training, in both the frail older subjects and healthy older subjects, suggesting that frail and pre-frail individuals have sufficient adaptive reserves to respond to training, despite the frail phenotype. This change towards healthier muscle is also reflected in the transcriptome. Not only do their muscles become stronger and larger, but this change is also evident at a molecular level.

The predictive model built in chapter 2 highlights genes that are linked to muscle function in frail older adults, healthy older adults and young men. This analysis revealed that the protocadherin gamma gene cluster showed a correlation with muscle strength. We suspected that this gene cluster was related to neuromuscular function, since this set of genes is involved in axon guidance. Muscle innervation is of vital importance for muscle function. While the protocadherin gamma gene cluster was a quite robust predictor of muscle strength, several other genes, functionally largely unknown, may also be important for muscle health and are worth highlighting here.

First, *ubiquitin specific peptidase 54* (USP54) is a gene that consistently popped up in the various comparisons discussed in this thesis (figure 7.1). Not only is it associated with muscle strength (table 2.6), training, age and frailty, but it is also upregulated by knee immobilization and downregulated by creatine supplementation during knee immobilization (figure 7.1). USP54 is a deubiquinating enzyme. The role of this enzyme is unknown within skeletal muscle, but it is associated with cancer progression, suggesting it may be involved in cellular growth an proliferation [2]. Second is *cilia and flagella associated protein 61* (CFAP61). This gene is also linked to muscle strength, age, frailty, training and knee immobilization. As briefly discussed in chapter 2, the function of this gene is still unknown, but may be associated with calcium metabolism. Lastly there is *dishevelled associated activator of morphogenesis 2* (DAAM2). This gene is part of the Wnt signalling pathway and, like USP54 and CFAP61, is associated with strength, age, training and knee immobilization. This gene is also mentioned the literature: in the context of muscle age [3], demyelination [4] and neuromuscular degeneration associated with Huntington's disease [5]. Since Wnt signalling has previously been associated with muscle aging and muscle fibrosis [6], this gene in particular may be interesting for future study.

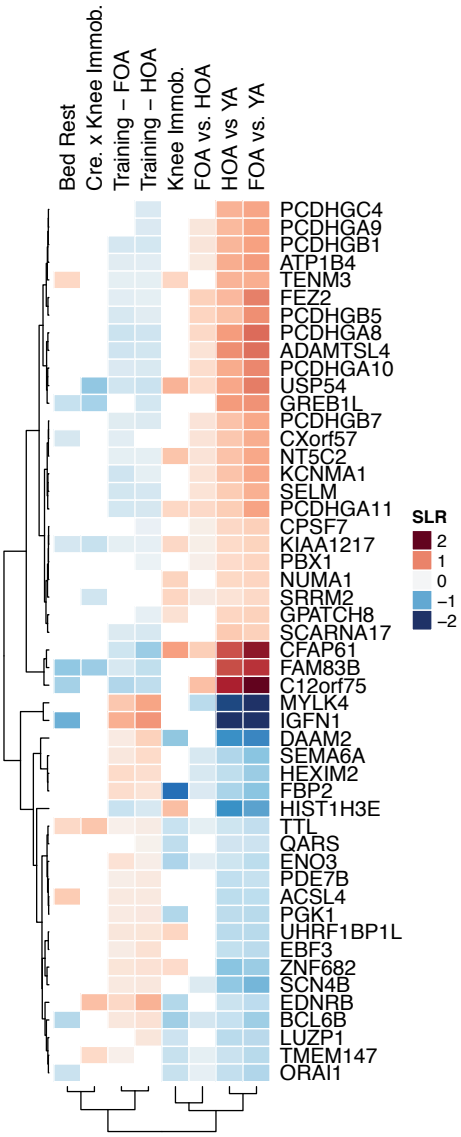

Figure 7.1 – Heatmap of the top 50 genes differentially expressed across the studies described in this thesis. SLR: signal log ratio. Cre: Creatine. FOA: Frail older adults. HOA: Healthy older adults. YA: Young adults.

In chapter 4 the role of vitamin D on the skeletal muscle transcriptome in frail older adults is investigated. Although there was no observable effect of vitamin D on the transcriptome, the population in this study overlaps with that of those in chapter 2 and 3. We also have muscle strength data from the participants of the vitamin D study, allowing us to also validate the predictive model from chapter 2. However, muscle strength data were obtained using different methods: BioDex and microFET. Both methods measure isometric maximal strength, whereas a dynamic strength test (1

repetition maximum) was used to train the predictive model in chapter 2. Moreover, newer array designs were used in chapter 4 (Affymetrix HuGene 2.1 vs. 1.1 ST arrays in chapter 2). Despite these methodological differences, the model we built to determine leg strength also showed predictive capabilities in the individuals in the vitamin D study, highlighting the robustness of the link between steady-state gene expression patterns and muscle strength and function (figure 7.2 and 7.3).

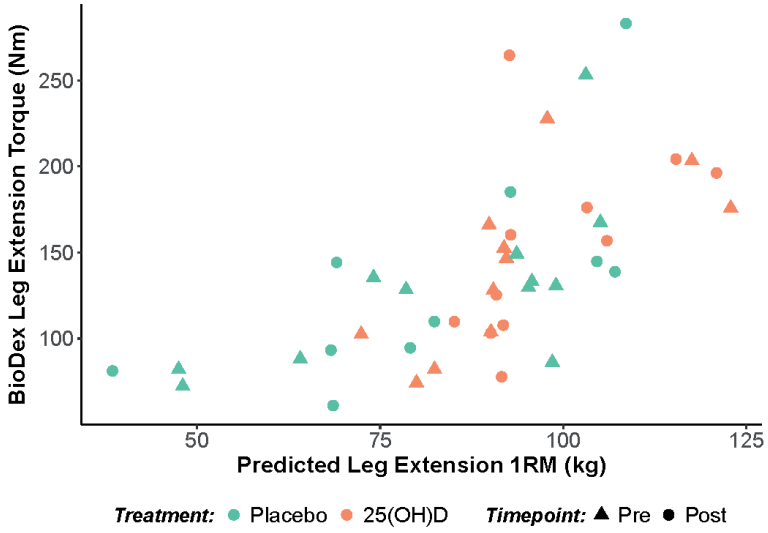

Figure 7.2 – Observed Biodex Leg Extension torque vs. predicted leg extension 1RM using the model described in chapter 2 in samples from the study described in chapter 5.

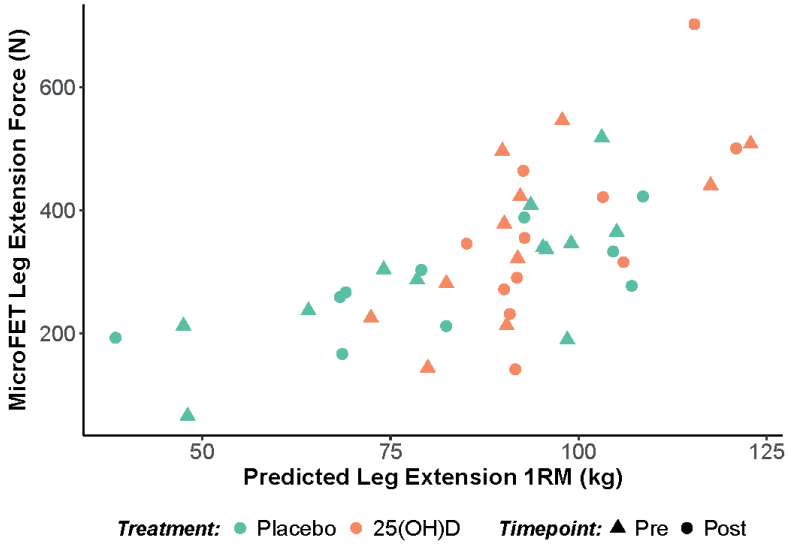

Figure 7.3 – Observed MicroFET leg extension force production vs. predicted leg extension 1RM using the model described in chapter 2 in samples from the study described in chapter 5.

Markers of muscle atrophy

While muscle strength appears to a have a robust signature in the muscle transcriptome, this does not seem to be the case for muscle size (i.e. muscle atrophy). In chapter 6 we found that the transcriptomic response to 7 days of bed rest and 7 days of knee immobilization are very dissimilar. Both models lead to similar (localized) rates of muscle atrophy [7], but this muscle atrophy is likely mediated via different mechanisms and regulators, as shown in chapter 6. Knee immobilization led to induction of histone deacetylase 4 / myogenin axis and proteasomal genes, whereas bed rest showed induction of autophagy and immune genes. The dissimilarity between these transcriptomic responses alone suggests that finding marker genes for muscle atrophy can prove challenging. Previous studies used mice to identify genes that are common to many types of muscle atrophy, leading to the widespread use of atrophy genes MAF-bx and Murf-1 (FBXO32 and TRIM63, respectively) [8-10]. However, these genes have shown to be inconsistently associated with muscle atrophy, particular in relation to age-related muscle loss [11, 12].

The goal of the two predictive sPLS models in chapter 6 was to identify genes that could be used as suitable markers for muscle atrophy in human tissues. One model, the naïve model, used all genes after filtering. This model identified ubiquitin-mediated proteolysis genes as the most reliable read-outs for the amount of muscle loss, but not FBXO32 and TRIM63. The second model used genes that were significantly changed in both models. This model showed higher predictive accuracy than the naïve model, but this may be due to 'double-dipping' (i.e. positively biasing the accuracy of the model by using pre-selected genes). The variable importance list for this model did reveal that Yippee Like 1 (YPEL1) was the most predictive of muscle loss in these two datasets (scatter plot of the change in YPEL1 expression and change in muscle size can be found in figure 7.4). The role of this gene is largely unknown, especially in the context of skeletal muscle. However, it may be involved in cell morphology and apoptosis [13, 14]. Cross-referencing this gene with the list of genes across all atrophy models from chapter 6 reveals that this gene is only significant in our bed rest and knee immobilization datasets. This illustrates the difficulty in finding suitable read-outs for muscle atrophy. However, YPEL1 may be an interesting candidate gene for further study in the context of muscle function and atrophy.

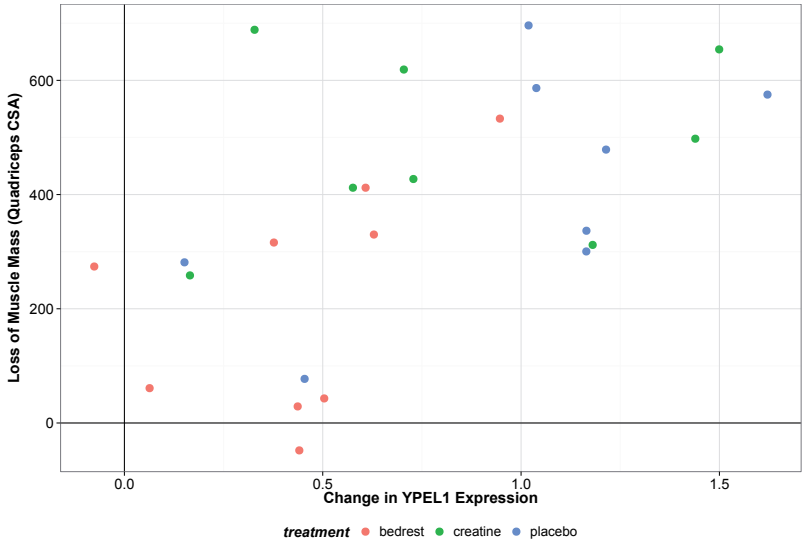

Figure 7.4 – Change in YPEL1 gene expression during muscle disuse (either knee immobilization or bed rest) compared with loss of muscle cross-sectional area (CSA) over the same period.

A possible explanation for this difficulty may be timing. Timing plays a crucial role in the assessment of molecular effects of various stimuli in skeletal muscle (or in this case, a lack of stimuli). Each gene has a different response profile to a certain stimulus, depending on its regulation and role. Some genes may peak immediately after a stimulus, while others can peak hours or even days after a stimulus (illustrated in figure 7.5). Others still may simply increase or decrease expression as an adaptation to repeated stimuli (f. ex. exercise training effect) or due to a lack of repeated stimuli (f. ex. detraining due to physical inactivity) [15]. While this is in the context of discrete stimuli such as an exercise session, duration of disuse may also play a role. Short disuse (such as one day or two days) may be characterized by different profiles of genes than longer duration (such as weeks or even months). The individual responses of genes can also be dependent on the status of the muscle. For example, detrained muscle is suspected to respond more severely to a single bout of exercise, whereas this stimulus would result in a negligible response in trained muscle (repeated bout effect) [16] .

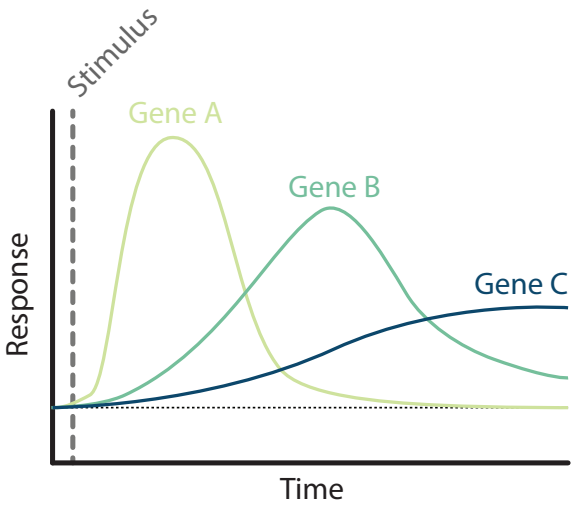

Figure 7.5 - Potential curves for gene expression in response to a stimulus, highlighting the importance of timing for sampling.

In this context, it is likely that different study populations, different models and different durations of disuse are characterized by different profiles of genes. This lack of consistency among atrophy-related genes is highlighted in figure 6.4. The most robust gene is a subunit of the acetyl-choline receptor (CHRNA1), which is unlikely to be a gene that mediates muscle protein breakdown. In this analysis, YPEL1 is ranked 294th as the most consistent, whereas the canonical muscle atrophy genes FBXO32 and TRIM63 are ranked 1696th and 4327th, respectively. As mentioned in chapter 6, some of the most interesting potential markers for muscle atrophy are *multiple EGF-like domain 10* (MEGF10) and *growth arrest and DNA damage inducible alpha* (GADD45A; ranked 13th and 93rd, respectively).

Countermeasures against sarcopenia

Age-related muscle loss develops over the course of a lifetime. As a consequence, it is a difficult process to study in intervention trials, particularly nutritional and exercise training trials where the effects are relatively small and accrue over longer periods. Chapter 6 (figure 6.3) shows that there is a significant overlap in the transcriptomic response to knee immobilization and the signature of age and frailty, whereas bed rest did not. This can also be seen to some extent in figure 7.1, where knee immobilization clusters with the age and frailty effects.

While using a disuse model to assess strategies as countermeasures for sarcopenia may not translate directly due to for example the differences in timeframe, there are some

compelling benefits to doing so. First, the relatively short timeframe and large effects for muscle disuse allows for faster screening of muscle atrophy countermeasures. Second, young volunteers are more readily recruited than frail or sarcopenic older adults. Lastly, countermeasures for muscle disuse can also indirectly prevent age-related muscle loss by attenuating muscle loss during disuse events (as outlined in figure 1.2). However, there is no guarantee that successful strategies that protect muscle size and strength during knee immobilization are also beneficial in the treatment of sarcopenia. This finding merely shows that knee immobilization or more prolonged unilateral lower limb suspension, based purely on the transcriptome, is the most suitable analogue for age-related muscle loss, whereas bed rest is the least suitable.

Since creatine seems to attenuate the changes that occur in the transcriptome due to knee immobilization, this may be a (very) tentative indication that creatine is beneficial for older individuals with regard to muscle loss. Others have cautiously have suggested that creatine supplementation may be beneficial in the context of sarcopenia [17]. Creatine is known to enhance muscle growth during resistance-type exercise training [18] and this effect is also seen among older individuals [19]. However, there is a lack of studies on the long term effect of creatine use on muscle size and function, especially in the context of sarcopenia [17].

Metabolomics in muscle research

Metabolomics allows for more detailed insight into energy metabolism. In particular, the relationship between amine levels and carnitine levels can shed light on amino acid oxidation, which might be especially relevant for muscle atrophy. Training led to a decrease in amino acid derived acyl carnitines, whereas amino acid levels were increased, implying a amino acid sparing effect. Conversely, we observed an increase in amino acid an amino acid derived acyl carnitine, even though the rest of the (fat derived) carnitines were decreased (chapter 4). These observations regarding amino acid oxidation were much less obvious in the transcriptome data.

The studies in both chapter 3 and chapter 4 also hinted that polyamine metabolism may also be linked to age and muscle disuse. Polyamines are especially interesting with regard to senescence due to their role in cell growth, survival and proliferation [20] and may even be useful as anti-aging compounds [21]. Various studies have also linked polyamine metabolism with muscle function [22]. The unique mechanism by which polyamines are regulated also highlights the importance of using multiple layers of ~omics simultaneously. Ornithine decarboxylase (ODC) activity is the main enzyme that regulates polyamine levels. Protein levels of ODC are regulated at the protein level via a negative feedback loop that is somewhat unique to polyamine metabolism [23]. As a consequence of this unique mechanism, changes in polyamine metabolism are particularly difficult to detect using transcriptomic techniques alone.

In chapter 3 we performed an integrative analysis of both the muscle metabolome and the transcriptome. While we found strong correlations within the tissue between the transcriptome and metabolome, the link with the metabolites and gene expression was likely indirect. The missing link between the transcriptome and metabolome is of course the proteome. Simultaneous measurement of the proteome and metabolome may also enhance the understanding of the link between the transcriptome and metabolome.

The weak correlations between plasma levels of metabolites with muscle levels of metabolites also highlights the importance of measuring the metabolome in the tissue itself. Myobolites, i.e. metabolites secreted by muscle that have a paracrine or systemic signalling role, have also been proposed as potentially interesting metabolites for further study [24]. However, even the putative myobolite β-aminoisobutyric acid, a metabolite suggested to promote fat oxidation in the liver [25], did not show strong correlations between muscle tissue and blood levels (chapter 3). These weak correlations could be due to the fact that the muscle was in a steady-state and it is possible that these myobolites can primarily be detected in an acute setting, such as during or immediately after exercise. Exploring the muscle metabolome immediately after or even during exercise might provide additional insights in the role of the muscle as a secretory organ.

Future Perspectives

The muscle transcriptome and metabolome can provide unique insights into muscle health. Frailty showed a clear signature in the muscle transcriptome and metabolome, largely independent of chronological age. We also observed a robust link between the muscle strength and transcriptome. There are also other links between the transcriptome and muscle health, such as physical activity (figure 7.6) and muscle fibre type distribution (figure 7.7). Together these outcomes can provide insight into an individual's lifestyle and overall health and perhaps pave the way towards personalized nutrition and exercise. Based on the transcriptome alone using a single sample we can estimate sex, age, frailty, muscle strength, muscle fiber type distribution and habitual physical activity. Other features, such as mitochondrial function, energy metabolism and various other indices can be contrasted using a reference dataset, such as in chapter 2 and 3. By providing a comprehensive overview of the health status of an individual's muscle with a single measurment we can perhaps guide dietary and physical activity recommendations to optimize muscle health.

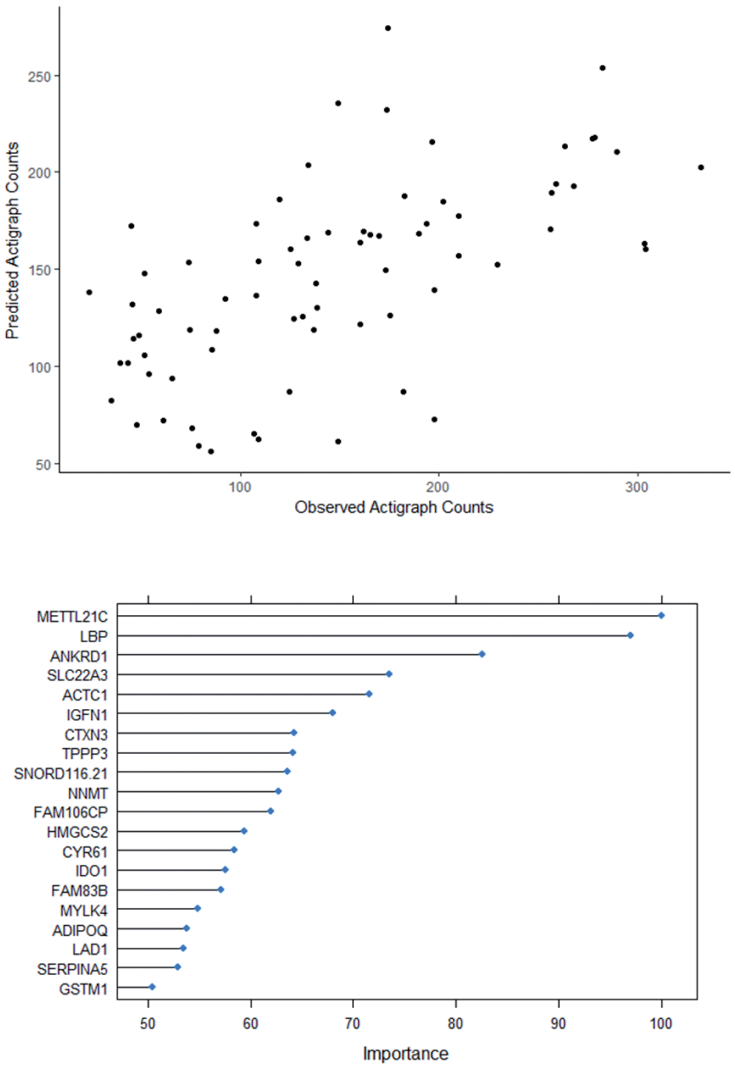

Figure 7.6 – Predictive model for general physical activity based on the transcriptome. Model was trained on ActiGraph data from the frail individuals (chapter 2 and 3). A (top): Observed vs. predicted actigraph counts (more means more physical activity). B (right): Top 20 predictor genes.

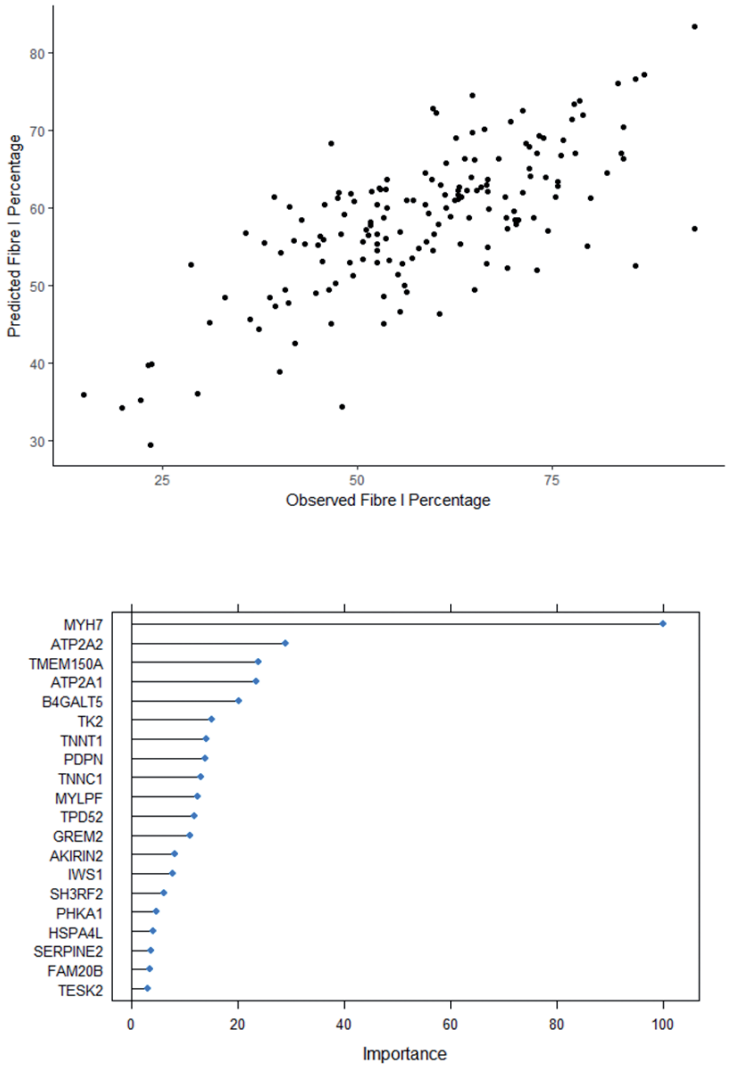

Figure 7.7A and B – Predictive model for muscle fibre type distribution based on the transcriptome. Model was trained on histological data from the frail and healthy older individuals (chapter 2 and 3). A (top): Observed vs. predicted type 1 fiber distribution. B (bottom): Top 20 predictor genes.

Before personalization based on ~omics profiling becomes feasible we would first need more detailed reference data. Comprehensive assessment of muscle function using a wide array of techniques (muscle performance, endurance, electromyography, histology, etc.) in addition to muscle biopsies would provide a large reference dataset to compare new samples to. Interventions, such as exercise, nutrition or pharmacological treatment, would have to be assessed both acutely and chronically to provide possible ways to shift the molecular profile of skeletal muscle in targeted directions. For example, protein supplementation could be proscribed based on the transcriptome. In chapter 6 one data set provides the transcriptomic signature of protein deficiency (figures 6.2 and 6.3) which would be recognized based on the gene expression profile within a sample, without needing nutritional diaries. Exercise could be proscribed based on for example predicted muscle strength or physical activity levels, mitochondrial function indices and/or fibre type distribution. Such an approach may be very valuable in populations such as frail older adults where the capacity to perform sufficiently intense exercise is limited.

Since muscle biopsies can lead to discomfort, it may be worthwhile to use muscle micro-biopsies rather than full Bergrström needle biopsies, as a relatively small amount of tissue is required for transcriptomics or metabolomics, and microbiopsies are more comfortable for subjects [26]. Another hurdle that needs to be passed in the use of these techniques for personalized exercise or nutrition is that of batch effects. Batch effects can significantly affect the results of ~omics techniques. While there are techniques to reduce batch effects, such as ComBat (which was employed to produce figures 7.2 and 7.3) or surrogate variable analysis [27], ideally control samples would be included in all batches to more accurately deal with batch effects. Other possible techniques to deal with batch effects are single sample normalization techniques such as fRMA, UPC or YuGene normalization [28-30].

Muscle function can be dramatically improved, even in frail older individuals, with resistance-type exercise training. However, consistent intense exercise takes a lot of effort and may not even be an option for some individuals. Nutrition can play a key role in maintaining muscle mass and function for these individuals. Protein and amino acids for example have been widely studied in this context. There are possibly many other nutritional compounds that may be of interest, such as ursolic acid, β-hydroxy-β-methyl butyrate and creatine [31]. Ideally, these nutritional compounds would also be evaluated with regard to their effects on the transcriptome or metabolome.

While these nutrients show potential as countermeasures for sarcopenia, not just individual nutrients are of interest. More recently there has been a shift towards defining dietary patterns rather than focusing on single foods and nutrients. The goal is to capture the complex interactions and synergies between foods, rather than investigating certain nutrients and foods in isolation [32]. An example of such a dietary pattern would be the Mediterranean diet, which has many potential health benefits, and is characterized, among other features, by a relatively low intake of animal protein and high intake of plant foods and unsaturated fat [33]. Ideally, nutritional interventions would also investigate the effects on skeletal muscle,

potentially also providing possible insights in their effects on the molecular processes in the skeletal muscle. Since frailty can roughly be regarded as the opposite of healthy aging, placing the effect of such a diet on the muscle transcriptome and metabolome in the context of the profiles discussed in this thesis might provide further insights of nutritional interventions with regard to healthy aging.

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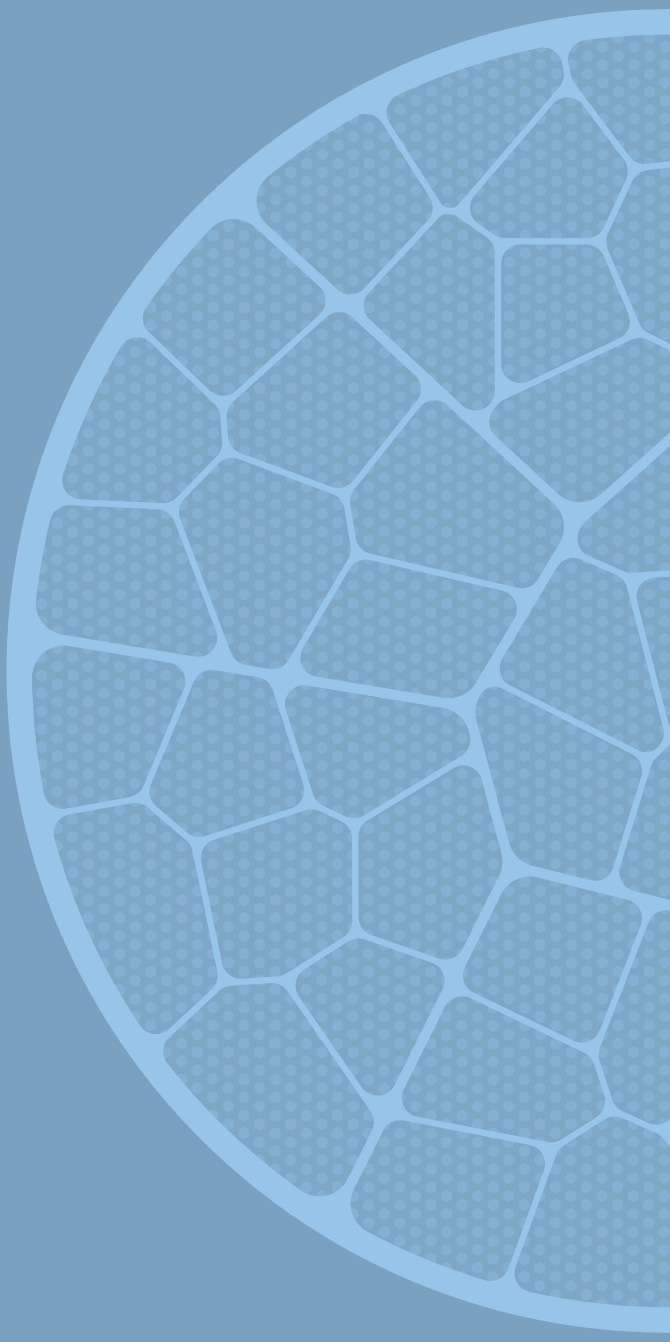
Supplementary materials



Chapter 2

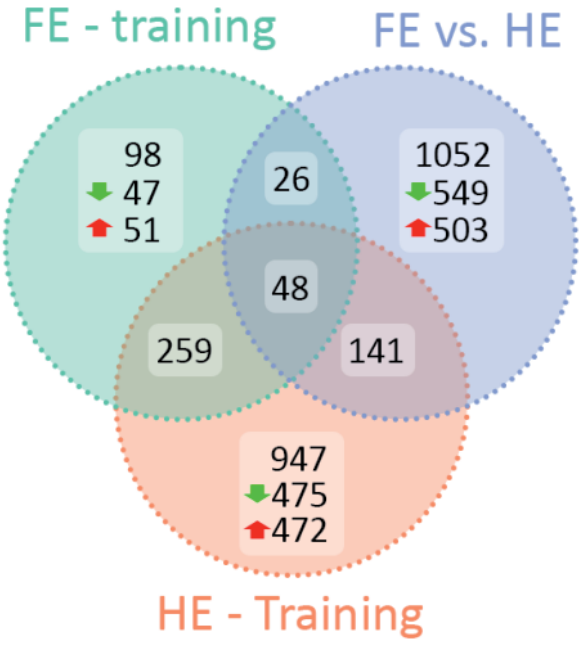


Figure S2.1A – Venn diagram of significant genes (q-value < 0.05) changed after prolonged exercise training in frail (FE – training, green) and healthy older subjects (HE – training, red). FE vs HE are genes significantly different at baseline between frail and healthy older subjects (blue).

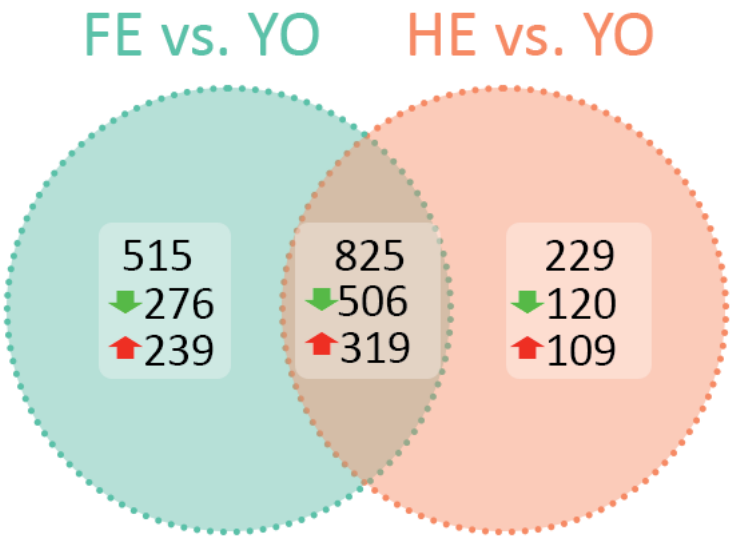


Figure S2.1B - Venn diagram of significant genes (q-value < 0.05) at baseline between the young (YO) group and both frail (FE) and healthy older subjects (HE). Due to the high number of significant genes with relatively small differences an additional fold-change cut-off was used for these data (fold change > 1.2).

Figure S2.2 – Top 40 (by p-value) significantly changed canonical pathways from Ingenuity Pathway Analysis. First two columns represent the prolonged exercise training effect in frail (FE) and healthy (HE) older subjects. Last three columns represent baseline differences between frail older subjects (FE), healthy older subjects (HE), and young subjects (YO). Colours indicate significance.

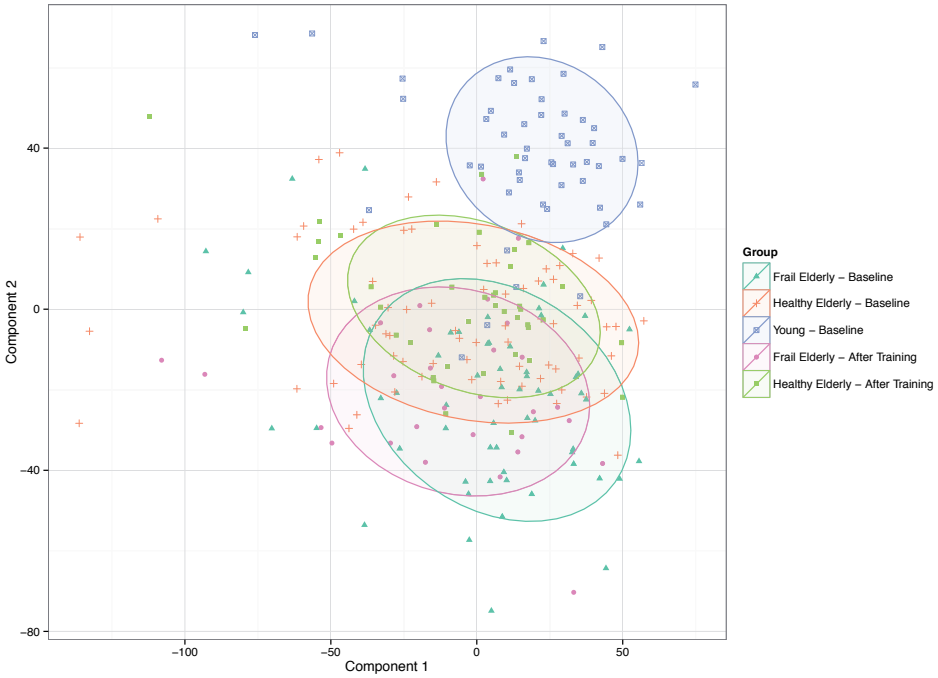


Figure S2.3 - PCA of all UPC filtered genes for all groups, before and after prolonged exercise training. Ellipses indicate 95% confidence interval.

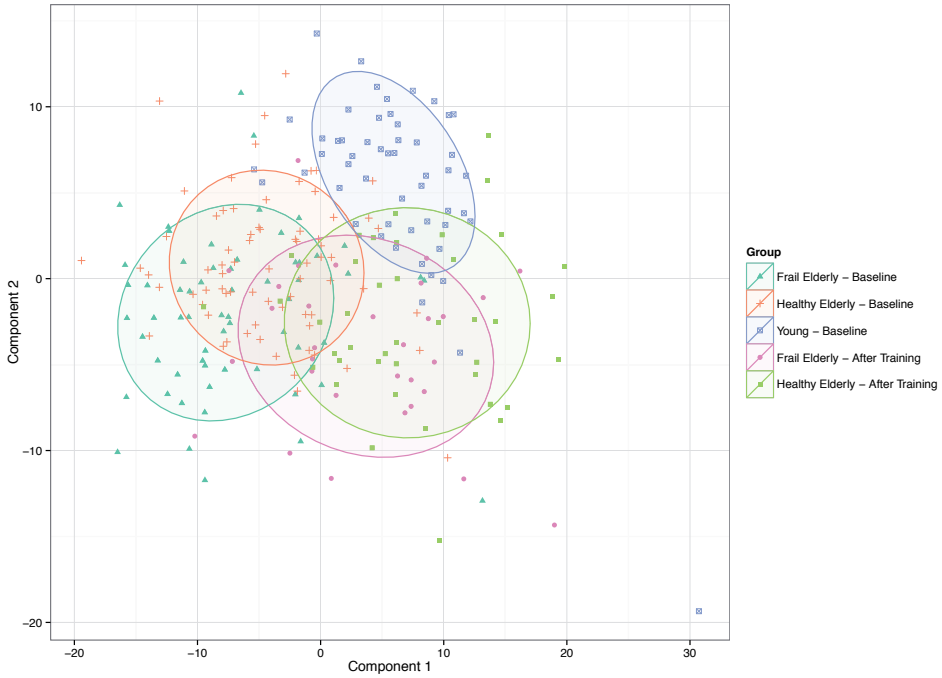


Figure S2.4 - PCA of 307 exercise responsive genes for all groups, before and after prolonged exercise training. Ellipses indicate 95% confidence interval.

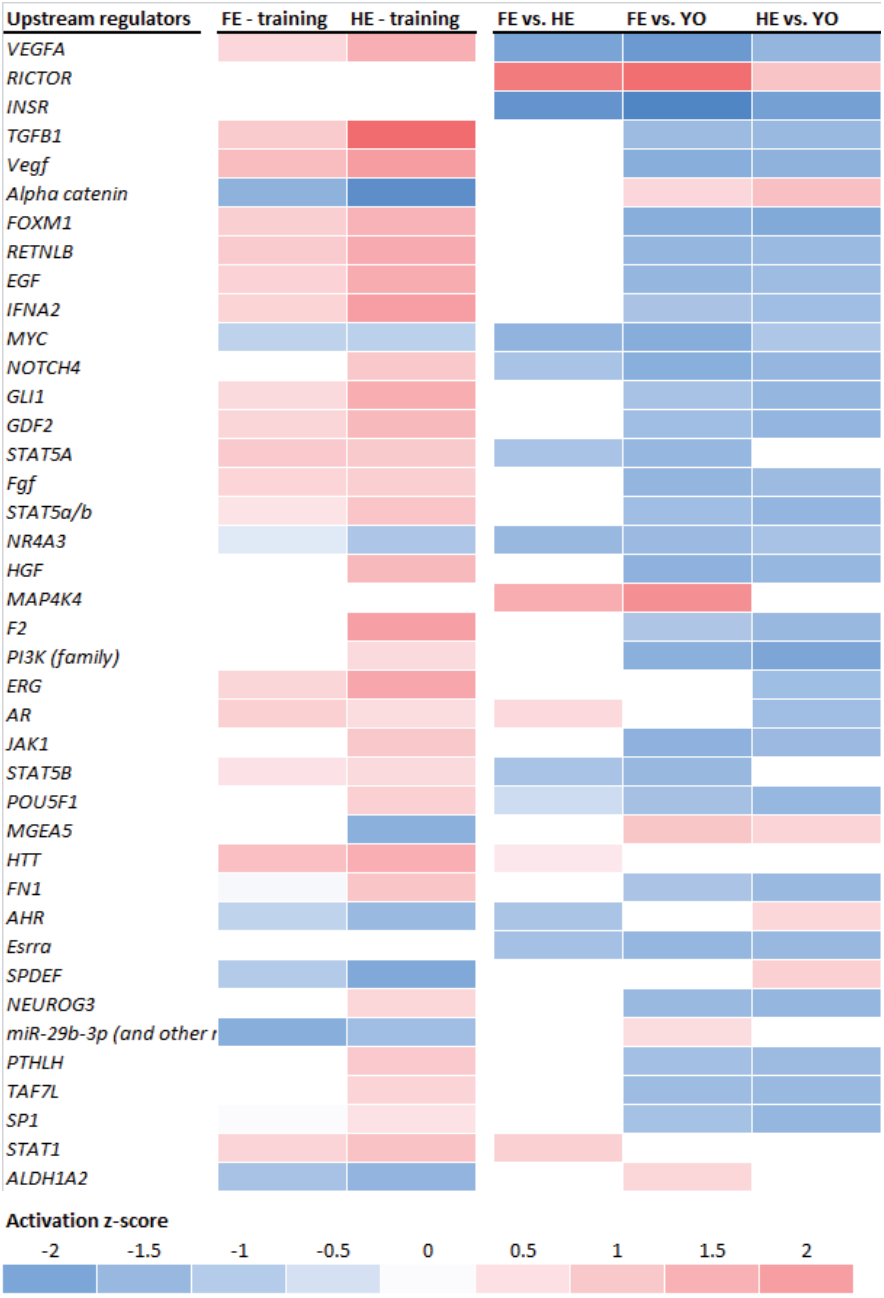


Figure S2.5 – Top 40 (by score) significant upstream regulators from Ingenuity Pathway Analysis. First two columns represent the prolonged exercise training effect in frail (FE) and healthy (HE) older subjects. Last three columns represent baseline differences between frail older subjects (FE), healthy older subjects (HE), and young subjects (YO). Colours indicate predicted activation.

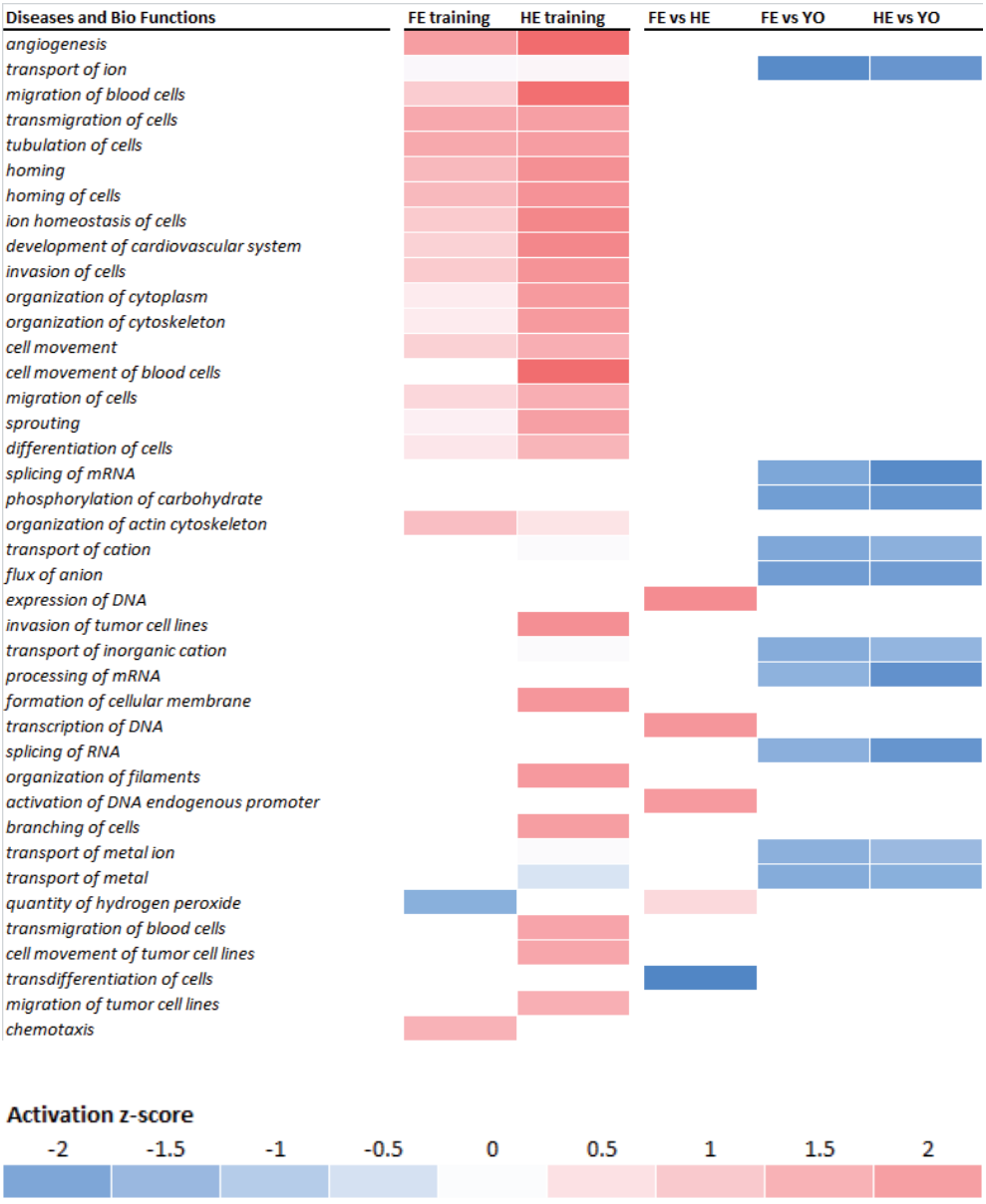


Figure S2.6 – Top 40 (by score) significantly changed diseases and functions from Ingenuity Pathway Analysis. First two columns represent the prolonged exercise training effect in frail (FE) and healthy (HE) older subjects. Last three columns represent baseline differences between frail older subjects (FE), healthy older subjects (HE), and young subjects (YO). Colours indicate predicted activation.

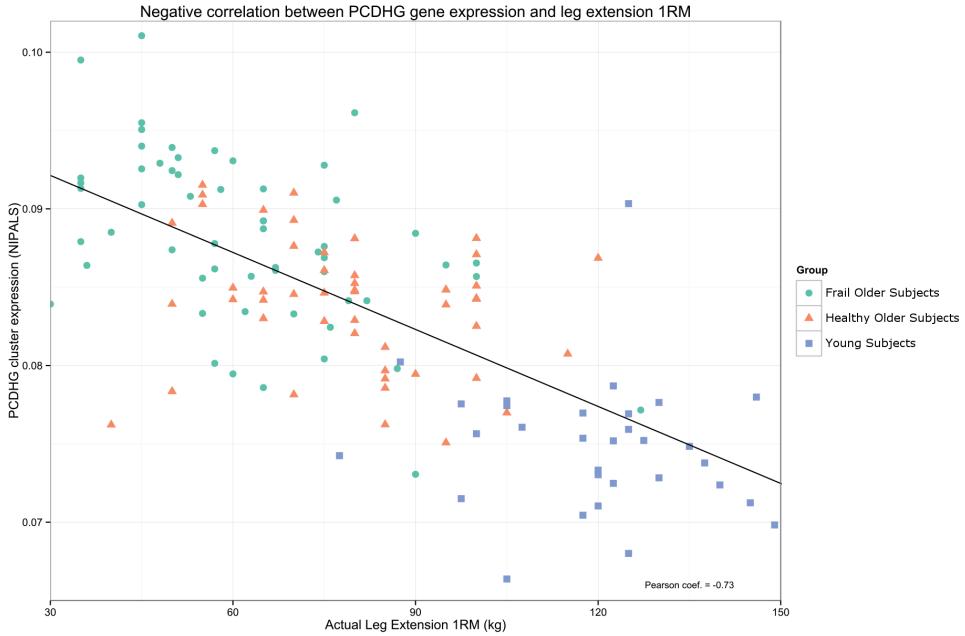


Figure S2.7 - Negative Correlation between the PCDHG gene cluster (eigengene as calculated using the NIPALS algorithm) and leg extension 1RM.

Chapter 3

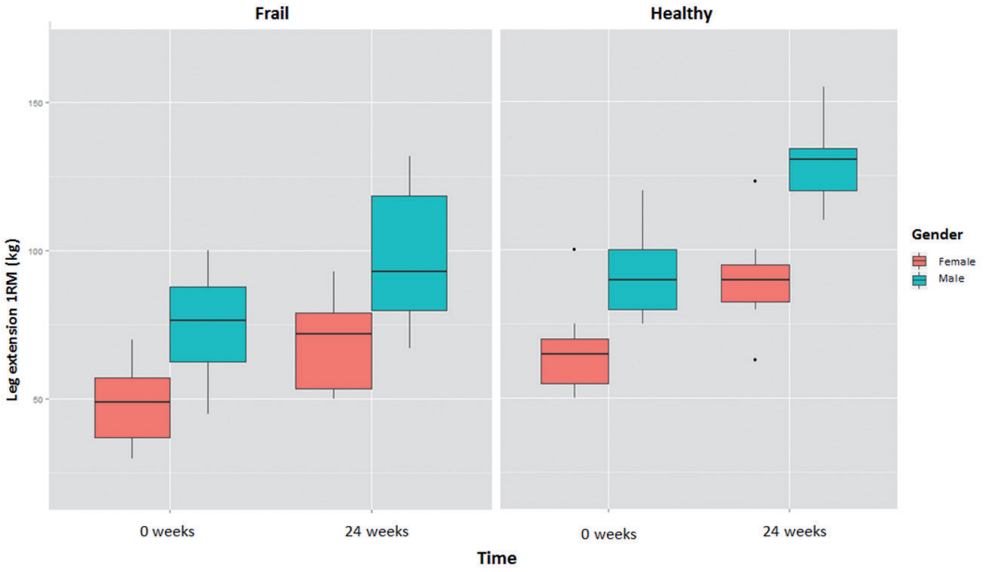


Figure S3.1 - Change of leg extension 1RM after resistance type exercise training. Left are frail older subjects, right are healthy older subjects.

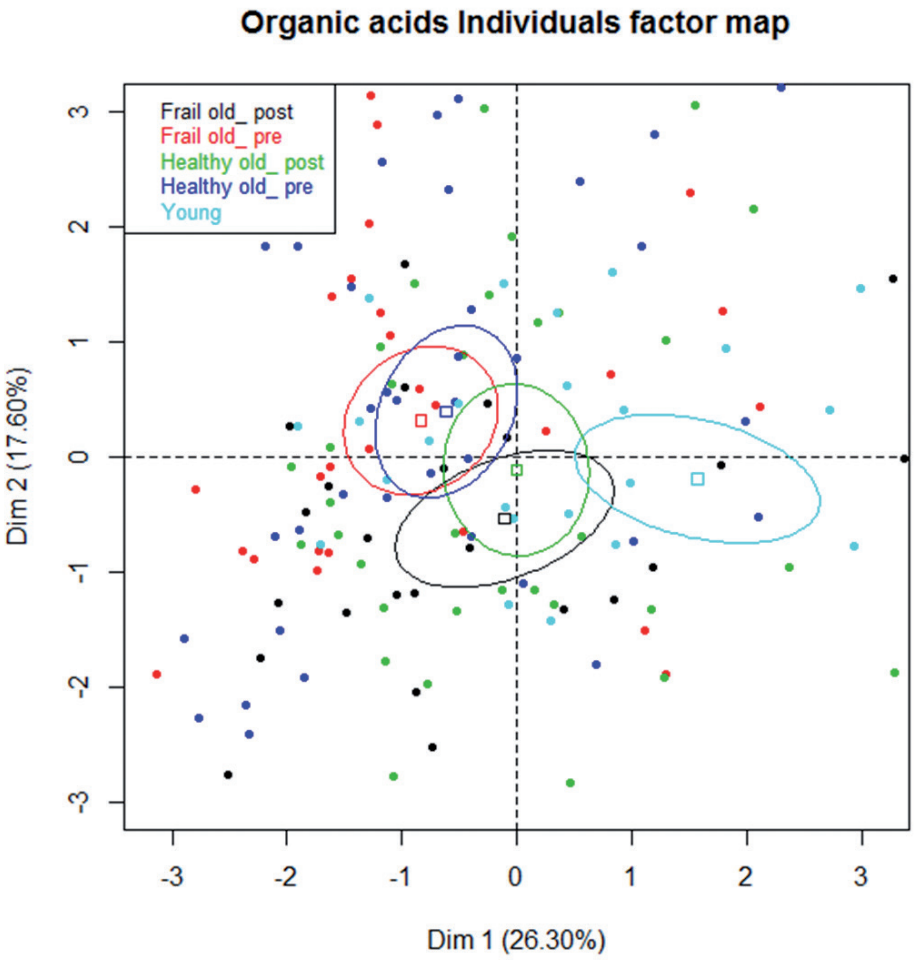


Figure S3.2. PCA plot of metabolites detected by the organic acid platform in muscle tissue. To visualize whether groups are significantly different from each other, confidence ellipses (95% confidence interval) were drawn around them. Post: after training, pre: before training.

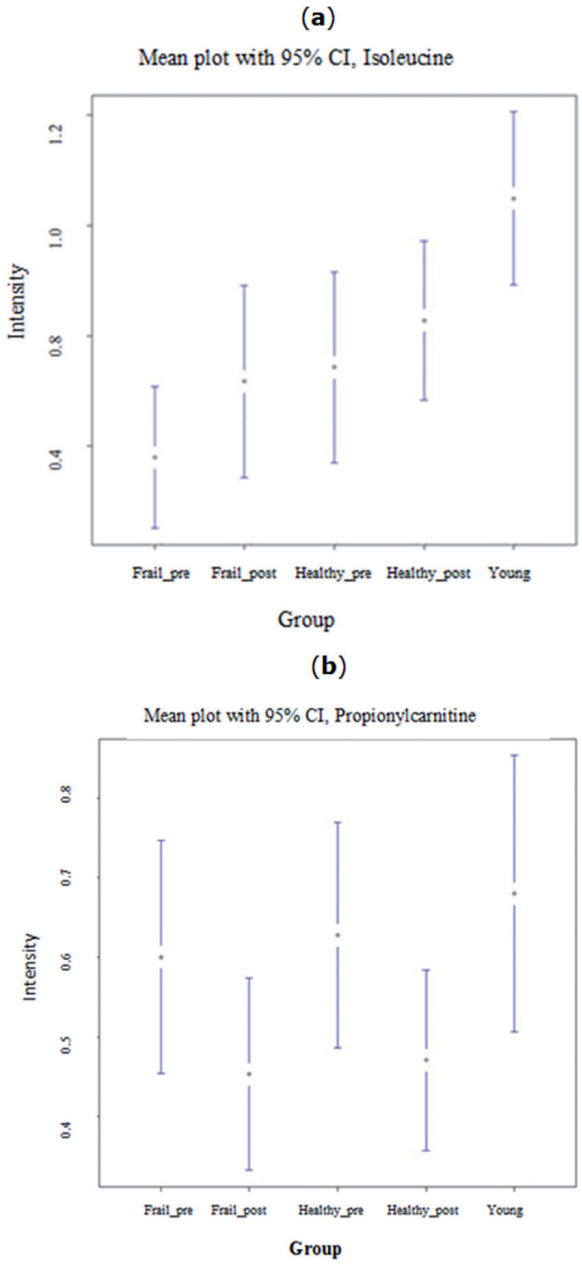


Figure S3.3 - Group means with 95 percent confidence intervals for (a) isoleucine and (b) propionylcarnitine. Standard deviations were relatively constant across groups. Frail_pre: frail older subjects before training, Frail_post: frail older subjects after training, Healthy_pre: healthy older subjects before training, Healthy_post: healthy older subjects after training.

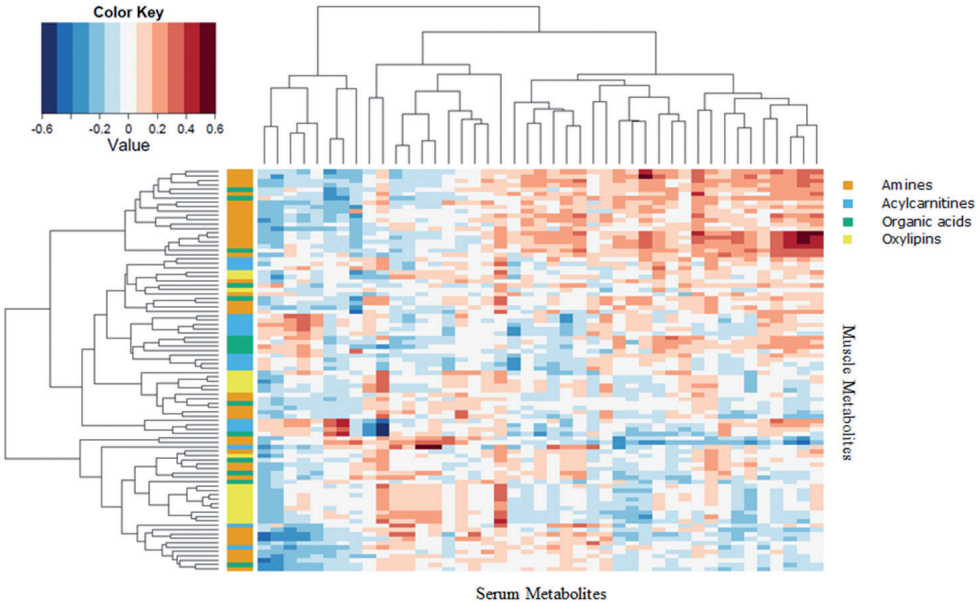


Figure S3.4 - Correlation heatmap muscle and serum metabolite. Pearson correlation were used. Red indicates positive correlation and blue indicates negative correlation.

Table S3.1 - Characteristics of subjects of which plasma samples were studied

	Young	Healthy older	Frail older
N (male / female)	50/0	51/25	35/27
Age (years)	21.7 ± 2.3	71.5 ± 5.1	78.1 ± 8.1
Height (m)	1.83 ± 0.05	1.71 ± 0.08	1.69 ± 0.09
Weight (kg)	76.4 ± 10.3	76.3 ± 12.9	79.2 ± 12.9
BMI[a] (kg / m^2)	23.0 ± 2.9	25.5 ± 3.0	27.8 ± 4.1
Body Fat (%)	15.4 ± 4.5	24.7 ± 5.7	32.66 ± 8.7

a:body mass index.

Table S3.2 - Characteristics of the older subjects with before and after training samples.

	Frail older pre[a]	Frail older post [b]	Healthy older pre	Healthy older post
N (male / female)	10/14		22/16	
Age (years)	76.0 ± 7.0		69.3 ± 4.0	
Height (m)	1.66 ± 0.09		1.70 ± 0.09	
Weight (kg)	77.7 ± 13.6	78.5 ± 14.0	75.9 ± 13.4	76.2 ± 13.6
BMI[c] (kg / m2)	27.9 ± 3.8	28.2 ± 3.9	26.0 ± 2.8	26.0 ± 2.8
Body Fat (%)	35.4 ± 8.3	35.2 ± 8.8	26.4 ± 5.4	24.7 ± 5.0*
Lean Mass (kg)	46.9 ± 9.9	47.4 ± 10.1*	53.6 ± 11.2	55.1 ± 11.5
Leg Extension 1RM (kg)	60.1 ± 18.9	82.7 ± 23.3*	79.8 ± 18.2	112.6 ± 22.5*
Leg Press 1RM (kg)	130.1± 33	176 ± 43.0*	175.9 ± 40.4	227.0 ± 49.9*

Chapter 4

Table S4.1 – Top 100 genes significantly changed according to main effect F-test (q-value < 0.05). FC is the signed fold change. Q-value is the p-value adjusted using Benjamini-Hochberg False Discovery Rate. Pla.: placebo group. Cre.: creatine group. Inter.: interaction effect. ES: Enrichment Score.

Symbol	FC Pla.	FC Cre.	FC Inter.	P-val Pla.	P-val Cre.	P-val Inter.	Q-val. F-test
ATXN1	1.61	1.24	-1.3	0	0.001	0.003	0.001
HDAC4	3.24	1.67	-1.94	0	0.006	0.01	0.002
IP6K3	2.32	1.73	-1.34	0	0	0.102	0.002
KDM7A	2.12	1.79	-1.19	0	0	0.331	0.002
YPEL1	1.96	1.81	-1.08	0	0	0.615	0.002
CALM1	1.71	1.69	-1.01	0	0	0.917	0.002
TULP4	1.63	1.36	-1.2	0	0.001	0.108	0.003
NMRK2	-5.23	-2	2.61	0	0.011	0.013	0.003
MTURN	1.51	1.33	-1.14	0	0.001	0.205	0.003
JMY	1.54	1.29	-1.2	0	0.002	0.08	0.004
PER2	1.47	1.17	-1.25	0	0.018	0.016	0.004
SLK	1.42	1.19	-1.2	0	0.008	0.038	0.004
SLC16A3	-2.65	-1.74	1.52	0	0.003	0.077	0.004
ABCA5	2.29	1.61	-1.42	0	0.003	0.091	0.004
MPP6	2.21	1.66	-1.34	0	0.002	0.146	0.004
VPS36	1.34	1.23	-1.09	0	0.001	0.255	0.004
HOOK3	1.33	1.26	-1.06	0	0	0.434	0.004
MAPRE3	1.7	1.53	-1.11	0	0	0.454	0.004
PIAS2	1.48	1.39	-1.06	0	0	0.565	0.004
SMAD4	1.31	1.26	-1.04	0	0	0.577	0.004
TBC1D15	1.42	1.5	1.06	0	0	0.594	0.004
FILIP1	1.52	1.12	-1.35	0	0.096	0.005	0.004
GDNF	4.64	2.56	-1.81	0	0.003	0.136	0.004
XRN1	1.52	1.34	-1.13	0	0.001	0.273	0.004
PPM1L	1.68	1.61	-1.04	0	0	0.772	0.004
POMK	1.47	1.42	-1.03	0	0	0.798	0.004
FAM160B1	1.4	1.43	1.03	0	0	0.812	0.004
CHMP1B	1.99	1.92	-1.04	0	0	0.863	0.004
IMPAD1	1.75	1.74	-1.01	0	0	0.961	0.004
DCP2	1.75	1.47	-1.19	0	0.002	0.254	0.004
ZC3H12C	1.47	1.27	-1.16	0	0.003	0.153	0.004
ERGIC1	-1.34	-1.25	1.07	0	0.001	0.4	0.004
SLC36A2	-1.67	-1.36	1.22	0	0.004	0.139	0.004
TRIM33	1.38	1.32	-1.05	0	0.001	0.631	0.004
RDX	1.28	1.17	-1.1	0	0.004	0.173	0.004
NRP1	-1.4	-1.28	1.09	0	0.001	0.342	0.004
ARPP21	2.25	1.23	-1.83	0	0.129	0.005	0.005

BCAR1	1.51	1.22	-1.24	0	0.013	0.05	0.005
GPI	-1.65	-1.31	1.26	0	0.008	0.082	0.005
DHRS7B	-1.45	-1.31	1.11	0	0.002	0.323	0.006
PNISR	1.38	1.09	-1.26	0	0.123	0.01	0.006
TP63	2.62	1.37	-1.91	0	0.068	0.012	0.006
OSBPL6	2.25	1.35	-1.67	0	0.05	0.02	0.006
CDK5RAP2	1.43	1.16	-1.23	0	0.028	0.037	0.006
ZC3H11A	1.48	1.22	-1.21	0	0.013	0.074	0.006
CHRNB1	1.66	1.3	-1.28	0	0.014	0.08	0.006
GSE1	1.38	1.18	-1.17	0	0.011	0.085	0.006
CLIP1	1.74	1.37	-1.27	0	0.008	0.129	0.006
PPIL4	1.65	1.36	-1.21	0	0.004	0.167	0.006
NFAT5	1.32	1.19	-1.11	0	0.005	0.172	0.006
TSR3	-1.4	-1.28	1.09	0	0.002	0.38	0.006
KMT2C	1.26	1.2	-1.05	0	0.002	0.469	0.006
UACA	1.42	1.34	-1.06	0	0.001	0.599	0.006
ANKRD12	1.35	1.31	-1.03	0	0.001	0.755	0.006
RICTOR	1.6	1.54	-1.04	0	0.001	0.781	0.006
RAD23B	1.31	1.28	-1.02	0	0.001	0.795	0.006
ATP9B	1.31	1.28	-1.02	0	0.001	0.813	0.006
TSPYL2	1.65	1.59	-1.03	0	0.001	0.842	0.006
AGO4	1.43	1.4	-1.02	0	0.001	0.852	0.006
EEF1D	-1.27	-1.27	-1	0.001	0.001	0.999	0.006
RBBP6	1.27	1.08	-1.18	0	0.075	0.013	0.006
UBE2H	1.36	1.21	-1.12	0	0.005	0.199	0.006
FAM46C	-2.32	-1.24	1.86	0	0.154	0.007	0.006
PARD3	1.44	1.16	-1.25	0	0.039	0.028	0.006
PFN2	1.98	1.32	-1.5	0	0.039	0.035	0.006
FGGY	1.78	1.29	-1.38	0	0.028	0.045	0.006
ACHE	-2.11	-1.59	1.32	0	0.006	0.199	0.006
RORA	2.01	1.55	-1.29	0	0.005	0.206	0.006
NEK7	1.6	1.35	-1.19	0	0.005	0.218	0.006
SLFN5	1.58	1.42	-1.11	0	0.002	0.451	0.006
CLOCK	1.43	1.35	-1.06	0	0.001	0.628	0.006
LMBRD2	1.29	1.33	1.03	0.001	0	0.754	0.006
SLC16A10	1.61	1.56	-1.03	0	0.001	0.834	0.006
TNKS	1.42	1.4	-1.01	0	0.001	0.936	0.006
UBE2W	1.63	1.67	1.02	0.001	0	0.887	0.006
EEA1	1.41	1.16	-1.22	0	0.036	0.041	0.006
AFF4	1.53	1.32	-1.17	0	0.005	0.227	0.006
DNAJC16	1.27	1.24	-1.03	0	0.001	0.75	0.006
EXOC2	1.46	1.21	-1.2	0	0.017	0.087	0.006
WDR59	1.36	1.18	-1.15	0	0.012	0.115	0.006
ABCA10	2.13	1.62	-1.31	0	0.006	0.227	0.006

SPG20	1.5	1.23	-1.22	0	0.016	0.095	0.007
FAM175A	1.9	1.31	-1.44	0	0.036	0.045	0.007
TXNL1	1.41	1.32	-1.07	0	0.002	0.569	0.007
EAPP	1.3	1.15	-1.14	0	0.017	0.107	0.007
IRF2BP2	-1.36	-1.22	1.11	0	0.006	0.245	0.007
FOXP2	1.64	1.42	-1.15	0	0.004	0.376	0.007
PHF20L1	1.27	1.2	-1.06	0	0.003	0.457	0.007
RFX5	1.38	1.29	-1.07	0	0.002	0.525	0.007
COL4A3	1.69	1.67	-1.01	0.001	0.001	0.936	0.007
SVIL	1.52	1.18	-1.29	0	0.051	0.034	0.007
NHLRC2	1.47	1.21	-1.22	0	0.021	0.087	0.007
HCFC2	1.3	1.19	-1.1	0	0.006	0.241	0.007
PLEKHA1	1.45	1.28	-1.13	0	0.005	0.288	0.007
SUPT6H	1.31	1.22	-1.07	0	0.004	0.425	0.007
TFRC	-2.45	-2.19	1.12	0	0.001	0.709	0.007
CSPP1	1.4	1.16	-1.21	0	0.041	0.051	0.007
GTF2A1	1.3	1.31	1.01	0.001	0.001	0.926	0.007
KDM5C	1.37	1.28	-1.07	0	0.003	0.499	0.007
MAP3K5	-1.37	-1.18	1.17	0	0.02	0.106	0.007

Supplementary table S4.2 – Top 50 enriched genesets results for the placebo group. Ranking based on Q-value. Q-value is the p-value adjusted using Benjamini-Hochberg False Discovery Rate. ES: Enrichment Score.

Name	Direction	ES	Q-value
REACTOME UNWINDING OF DNA	down	-0.8	0
REACTOME PYRUVATE METABOLISM	down	-0.7	0
REACTOME GLUCONEOGENESIS	down	-0.7	0
KEGG STEROID BIOSYNTHESIS	down	-0.7	0
PID IL8 CXCR2 PATHWAY	down	-0.7	0
PID IL8 CXCR1 PATHWAY	down	-0.7	0
REACTOME TRANSPORT OF RIBONUCLEOPROTEINS INTO THE HOST NUCLEUS	up	0.66	0
PID ALPHA SYNUCLEIN PATHWAY	down	-0.6	0
REACTOME DNA STRAND ELONGATION	down	-0.6	0
REACTOME PYRUVATE METABOLISM AND CITRIC ACID TCA CYCLE	down	-0.6	0
REACTOME THROMBIN SIGNALLING THROUGH PROTEINASE ACTIVATED RECEPTORS PARS	down	-0.6	0
REACTOME SIGNAL AMPLIFICATION	down	-0.6	0
REACTOME PEPTIDE CHAIN ELONGATION	down	-0.6	0
REACTOME GLUCOSE METABOLISM	down	-0.6	0
PID TXA2PATHWAY	down	-0.6	0
KEGG GLYCOLYSIS GLUCONEOGENESIS	down	-0.6	0
KEGG RIBOSOME	down	-0.5	0
REACTOME RESPIRATORY ELECTRON TRANSPORT ATP SYNTHESIS BY CHEMIOSMOTIC COUPLING AND HEAT PRODUCTION BY UNCOUPLING PROTEINS	down	-0.5	0
REACTOME TCA CYCLE AND RESPIRATORY ELECTRON TRANSPORT	down	-0.5	0
KEGG HEMATOPOIETIC CELL LINEAGE	down	-0.5	0
REACTOME IMMUNOREGULATORY INTERACTIONS BETWEEN A LYMPHOID AND A NON LYMPHOID CELL	down	-0.5	0
REACTOME SRP DEPENDENT COTRANSLATIONAL PROTEIN TARGETING TO MEMBRANE	down	-0.5	0
REACTOME INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	down	-0.5	0
PID TCR PATHWAY	down	-0.5	0
KEGG OXIDATIVE PHOSPHORYLATION	down	-0.5	0
KEGG SYSTEMIC LUPUS ERYTHEMATOSUS	down	-0.5	0
KEGG PARKINSONS DISEASE	down	-0.5	0
KEGG ALZHEIMERS DISEASE	down	-0.4	0
REACTOME ADP SIGNALLING THROUGH P2RY1	down	-0.6	0.002
KEGG LYSOSOME	down	-0.4	0.002
REACTOME RESPIRATORY ELECTRON TRANSPORT	down	-0.5	0.002
REACTOME PD1 SIGNALING	down	-0.7	0.004
REACTOME PYRIMIDINE METABOLISM	down	-0.6	0.004
KEGG CELL ADHESION MOLECULES CAMS	down	-0.4	0.004
KEGG PENTOSE PHOSPHATE PATHWAY	down	-0.6	0.004
BIOCARTA THELPER PATHWAY	down	-0.8	0.004

REACTOME CHOLESTEROL BIOSYNTHESIS	down	-0.6	0.004
BIOCARTA SRCRPTP PATHWAY	down	-0.8	0.004
REACTOME GENERIC TRANSCRIPTION PATHWAY	up	0.43	0.005
REACTOME PROSTACYCLIN SIGNALLING THROUGH PROSTACYCLIN RECEPTOR	down	-0.6	0.007
REACTOME TRANSPORT OF MATURE TRANSCRIPT TO CYTOPLASM	up	0.58	0.007
PID AMB2 NEUTROPHILS PATHWAY	down	-0.5	0.007
REACTOME THROMBOXANE SIGNALLING THROUGH TP RECEPTOR	down	-0.6	0.007
REACTOME HYALURONAN METABOLISM	down	-0.7	0.007
REACTOME FORMATION OF ATP BY CHEMIOSMOTIC COUPLING	down	-0.7	0.007
REACTOME METABOLISM OF CARBOHYDRATES	down	-0.4	0.008
REACTOME GLYCOLYSIS	down	-0.6	0.008
KEGG COMPLEMENT AND COAGULATION CASCADES	down	-0.5	0.008
BIOCARTA PROTEASOME PATHWAY	up	0.65	0.013
BIOCARTA MCM PATHWAY	down	-0.6	0.013
KEGG AUTOIMMUNE THYROID DISEASE	down	-0.5	0.014

Supplementary table S4.3 – Top 50 enriched genesets results for the interaction effect (immobilization × creatine supplementation). Ranking based on Q-value. Q-value is the p-value adjusted using Benjamini-Hochberg False Discovery Rate.

Name	Direction	ES	Q-value
PID IL8 CXCR2 PATHWAY	up	0.68	0
PID IL8 CXCR1 PATHWAY	up	0.65	0
KEGG LYSOSOME	up	0.62	0
BIOCARTA VITCB PATHWAY	up	0.76	0.003
REACTOME SIGNALING BY NOTCH4	up	0.77	0.003
REACTOME ACTIVATED NOTCH1 TRANSMITS SIGNAL TO THE NUCLEUS	up	0.62	0.003
PID ALPHA SYNUCLEIN PATHWAY	up	0.59	0.003
REACTOME THE ROLE OF NEF IN HIV1 REPLICATION AND DISEASE PATHOGENESIS	up	0.63	0.004
PID P75 NTR PATHWAY	up	0.49	0.004
REACTOME SRP DEPENDENT COTRANSLATIONAL PROTEIN TARGETING TO MEMBRANE	up	0.47	0.004
PID BCR 5PATHWAY	up	0.51	0.005
REACTOME ASPARAGINE N LINKED GLYCOSYLATION	up	0.49	0.005
REACTOME POL SWITCHING	up	0.77	0.006
REACTOME REGULATED PROTEOLYSIS OF P75NTR	up	0.84	0.006
REACTOME NRIF SIGNALS CELL DEATH FROM THE NUCLEUS	up	0.75	0.007
KEGG GLYCOSAMINOGLYCAN DEGRADATION	up	0.68	0.009
REACTOME RESPIRATORY ELECTRON TRANSPORT	down	-0.5	0.01
REACTOME RESPIRATORY ELECTRON TRANSPORT ATP SYNTHESIS BY CHEMIOSMOTIC COUPLING AND HEAT PRODUCTION BY UNCOUPLING PROTEINS	down	-0.5	0.01
PID TXA2PATHWAY	up	0.53	0.01
REACTOME MHC CLASS II ANTIGEN PRESENTATION	up	0.45	0.01
REACTOME LAGGING STRAND SYNTHESIS	up	0.62	0.01
PID GMCSF PATHWAY	up	0.51	0.011
BIOCARTA RAS PATHWAY	up	0.58	0.011
REACTOME SIGNALING BY NOTCH2	up	0.76	0.011
REACTOME SIGNALING BY ILS	up	0.42	0.011
REACTOME CD28 DEPENDENT VAV1 PATHWAY	up	0.73	0.011
PID ERBB1 DOWNSTREAM PATHWAY	up	0.42	0.011
REACTOME FORMATION OF THE TERNARY COMPLEX AND SUBSEQUENTLY THE 43S COMPLEX	up	0.5	0.011
BIOCARTA ACTINY PATHWAY	up	0.6	0.012
REACTOME POST TRANSLATIONAL PROTEIN MODIFICATION	up	0.4	0.012
REACTOME TRANSLATION	up	0.4	0.012
REACTOME SIGNALING BY NOTCH3	up	0.74	0.012
REACTOME N GLYCAN TRIMMING IN THE ER AND CALNEXIN CALRETICULIN CYCLE	up	0.69	0.012
REACTOME ACTIVATION OF THE MRNA UPON BINDING OF THE CAP BINDING COMPLEX AND EIFS AND SUBSEQUENT BINDING TO 43S	up	0.48	0.012

PID P38 ALPHA BETA PATHWAY	up	0.54	0.013
KEGG PENTOSE PHOSPHATE PATHWAY	up	0.58	0.013
KEGG B CELL RECEPTOR SIGNALING PATHWAY	up	0.43	0.013
KEGG VIBRIO CHOLERAE INFECTION	up	0.48	0.013
REACTOME CALNEXIN CALRETICULIN CYCLE	up	0.71	0.013
REACTOME DNA STRAND ELONGATION	up	0.61	0.013
REACTOME LYSOSOME VESICLE BIOGENESIS	up	0.6	0.013
BIOCARTA EDG1 PATHWAY	up	0.57	0.013
REACTOME SPHINGOLIPID METABOLISM	up	0.46	0.014
KEGG PATHOGENIC ESCHERICHIA COLI INFECTION	up	0.48	0.014
PID TCR PATHWAY	up	0.47	0.014
PID TOLL ENDOGENOUS PATHWAY	up	0.59	0.014
BIOCARTA CDC42RAC PATHWAY	up	0.64	0.014
REACTOME GLYCOLYSIS	up	0.58	0.014
REACTOME UNWINDING OF DNA	up	0.72	0.015
KEGG OTHER GLYCAN DEGRADATION	up	0.64	0.015

Chapter 5

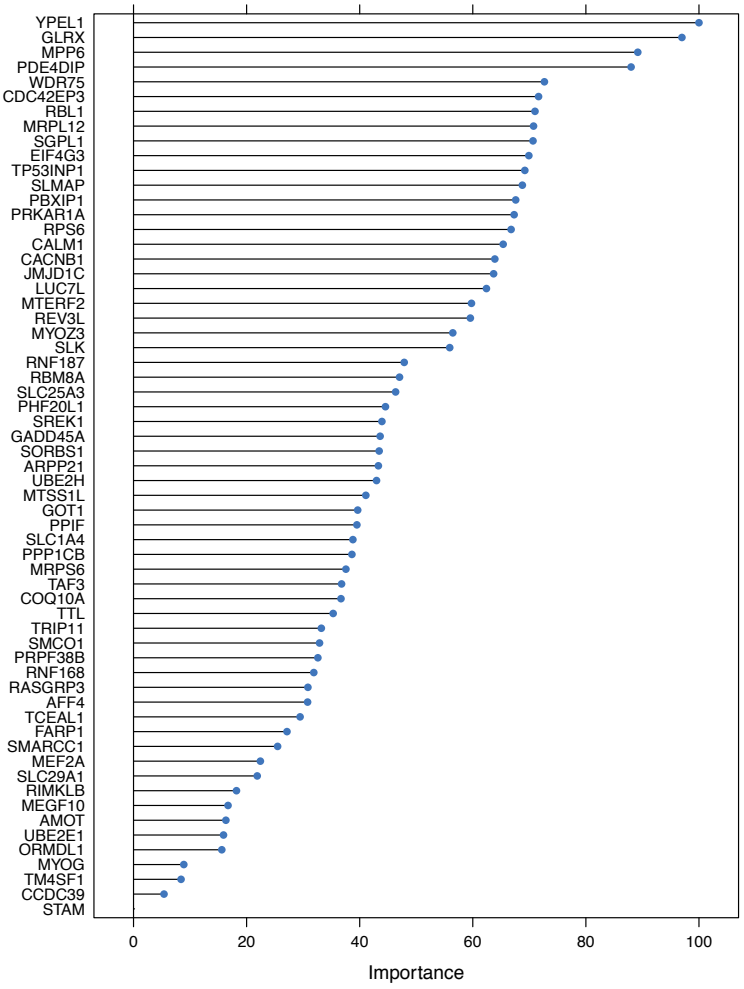
Supplementary Table S5.1 – Canonical pathways (from Ingenuity Pathway Analysis) regulated in the calcifediol group

Ingenuity Canonical Pathways	-log(p-value)	Ratio
CCR5 Signaling in Macrophages	2.03	0.143
OX40 Signaling Pathway	1.98	0.136
Guanine and Guanosine Salvage I	1.67	1
Methylglyoxal Degradation VI	1.67	1
Autoimmune Thyroid Disease Signaling	1.67	0.182
Graft-versus-Host Disease Signaling	1.67	0.182
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	1.67	0.182
UVA-Induced MAPK Signaling	1.6	0.0755
Superpathway of Cholesterol Biosynthesis	1.53	0.154
Allograft Rejection Signaling	1.47	0.143
Myc Mediated Apoptosis Signaling	1.43	0.0857
STAT3 Pathway	1.4	0.0833
Hematopoiesis from Pluripotent Stem Cells	1.38	0.5
Histidine Degradation III	1.38	0.5
Adenine and Adenosine Salvage III	1.38	0.5
S-adenosyl-L-methionine Biosynthesis	1.38	0.5
Type I Diabetes Mellitus Signaling	1.34	0.0789
Retinoic acid Mediated Apoptosis Signaling	1.31	0.118
Regulation of eIF4 and p70S6K Signaling	1.24	0.0505

Supplementary Table S5.2 – Upstream regulators (from Ingenuity Pathway Analysis) regulated in the calcifediol group

Upstream Regulator	Molecule Type	Activation z-score	p-value of overlap
CD247	transmembrane receptor	-1	0.00117
HOXA10	transcription regulator	-1.633	0.00187
SLC4A1	transporter		0.00252
DIRAS3	enzyme		0.00414
MYB	transcription regulator		0.00698
SREBF2	transcription regulator		0.0102
PRDM1	transcription regulator	-1	0.0109
PARP9	enzyme		0.0111
SIRT2	transcription regulator		0.0111
PTP4A3	phosphatase		0.0141
B2M	transmembrane receptor		0.0174
TR-RXR	complex		0.0208
HLA-B27	complex		0.0208
PARG	enzyme		0.0208
CHFR	enzyme		0.0208
HSPE1	enzyme		0.0208
PAM	enzyme		0.0208
FUT4	enzyme		0.0208
MPG	enzyme		0.0208
Ryr	group		0.0208

Chapter 6



Supplementary Figure S6.1 – Variable importance for sPLS model using common atrophy genes.

Supplementary Table S6.1 – Top 50 genesets enriched during knee immobilization. Ranking is based on absolute normalized enrichment score (NES). ES: Enrichment score. NES: Normalized enrichment score. NOM p-val: nominal p-value. FDR q-val: P-value adjusted using Benjamini-Hochberg False Discovery Rate.

NAME	SIZE	ES	NES	NOM p-val	FDR q-val
REACTOME TCA CYCLE AND RESPIRATORY ELECTRON TRANSPORT	116	-0.54408	-2.54447	0	0
REACTOME GLUCONEOGENESIS	29	-0.71399	-2.52042	0	0
PID IL8 CXCR2 PATHWAY	33	-0.66926	-2.50223	0	0
REACTOME RESPIRATORY ELECTRON TRANSPORT ATP SYNTHESIS BY CHEMIOSMOTIC COUPLING AND HEAT PRODUCTION BY UNCOUPLING PROTEINS	80	-0.55314	-2.42203	0	0
KEGG SYSTEMIC LUPUS ERYTHEMATOSUS	115	-0.50963	-2.387	0	2.73E-04
REACTOME PYRUVATE METABOLISM AND CITRIC ACID TCA CYCLE	40	-0.60776	-2.33714	0	7.21E-04
REACTOME PEPTIDE CHAIN ELONGATION	73	-0.54813	-2.32228	0	6.18E-04
KEGG OXIDATIVE PHOSPHORYLATION	115	-0.50111	-2.30176	0	5.41E-04
KEGG RIBOSOME	76	-0.53375	-2.29167	0	4.81E-04
REACTOME SIGNAL AMPLIFICATION	31	-0.64852	-2.26112	0	5.84E-04
REACTOME SRP DEPENDENT COTRANSLATIONAL PROTEIN TARGETING TO MEMBRANE	94	-0.50862	-2.25438	0	5.31E-04
KEGG HEMATOPOIETIC CELL LINEAGE	83	-0.52008	-2.25351	0	4.86E-04
REACTOME ADP SIGNALLING THROUGH P2RY1	25	-0.66627	-2.25214	0	4.49E-04
PID ALPHA SYNUCLEIN PATHWAY	33	-0.60485	-2.24982	0	4.17E-04
PID TXA2PATHWAY	57	-0.55088	-2.24324	0	3.89E-04
REACTOME DNA STRAND ELONGATION	30	-0.64397	-2.24163	0	3.65E-04
REACTOME UNWINDING OF DNA	11	-0.83358	-2.2385	0	3.43E-04
REACTOME GLUCOSE METABOLISM	61	-0.54726	-2.22856	0	4.10E-04
KEGG PARKINSONS DISEASE	111	-0.47853	-2.21041	0	4.67E-04
REACTOME PYRUVATE METABOLISM	18	-0.72889	-2.19492	0	6.75E-04
PID IL8 CXCR1 PATHWAY	27	-0.64607	-2.19158	0	6.43E-04
REACTOME TRANSPORT OF MATURE TRANSCRIPT TO CYTOPLASM	50	0.610673	2.191445	0	6.91E-04
REACTOME FORMATION OF ATP BY CHEMIOSMOTIC COUPLING	13	-0.77339	-2.16842	0	8.89E-04
KEGG GLYCOLYSIS GLUCONEOGENESIS	59	-0.52751	-2.16788	0	8.51E-04
REACTOME INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	89	-0.49011	-2.15837	0	0.001004
REACTOME THROMBIN SIGNALLING THROUGH PROTEINASE ACTIVATED RECEPTORS PARS	32	-0.61208	-2.15799	0	9.64E-04
BIOCARTA PROTEASOME PATHWAY	28	0.684648	2.150185	0	0.001067

KEGG PENTOSE PHOSPHATE PATHWAY	26	-0.62511	-2.1495	0	0.001043
REACTOME IMMUNOREGULATORY INTERACTIONS BETWEEN A LYMPHOID AND A NON LYMPHOID CELL	59	-0.51203	-2.13955	0	0.001117
REACTOME THROMBOXANE SIGNALLING THROUGH TP RECEPTOR	23	-0.66108	-2.13288	0	0.001244
KEGG ALZHEIMERS DISEASE	154	-0.44412	-2.12922	0	0.001253
PID TCR PATHWAY	66	-0.50954	-2.11823	0	0.001414
BIOCARTA SRCRPTP PATHWAY	11	-0.80383	-2.1043	0	0.002058
REACTOME PROSTACYCLIN SIGNALLING THROUGH PROSTACYCLIN RECEPTOR	19	-0.66988	-2.09827	0	0.002272
REACTOME G BETA GAMMA SIGNALLING THROUGH PLC BETA	20	-0.6722	-2.09201	0	0.002488
REACTOME DEPOSITION OF NEW CENPA CONTAINING NUCLEOSOMES AT THE CENTROMERE	55	-0.50423	-2.09116	0	0.002415
REACTOME PYRIMIDINE METABOLISM	23	-0.63739	-2.08683	0	0.002473
KEGG GRAFT VERSUS HOST DISEASE	36	-0.56536	-2.07124	0	0.002959
KEGG STEROID BIOSYNTHESIS	16	-0.69675	-2.05679	0.002364	0.003783
KEGG GLYCOSPHINGOLIPID BIOSYNTHESIS GLOBO SERIES	14	-0.72102	-2.05459	0	0.003723
REACTOME RESPIRATORY ELECTRON TRANSPORT	64	-0.49993	-2.03772	0	0.004484
REACTOME TRANSPORT OF RIBONUCLEOPROTEINS INTO THE HOST NUCLEUS	26	0.666511	2.035447	0	0.012914
REACTOME ADP SIGNALLING THROUGH P2RY12	21	-0.62767	-2.01733	0	0.005873
KEGG LYSOSOME	119	-0.43433	-2.0069	0	0.00661
REACTOME G PROTEIN BETA GAMMA SIGNALLING	28	-0.58087	-2.00357	0	0.006738
REACTOME METABOLISM OF CARBOHYDRATES	226	-0.3909	-2.00255	0	0.006647
KEGG PROTEASOME	43	0.578985	2.001636	0	0.015836
KEGG COMPLEMENT AND COAGULATION CASCADES	67	-0.47893	-1.99819	0	0.006778
REACTOME G1 S SPECIFIC TRANSCRIPTION	16	-0.66605	-1.9956	0	0.006798
REACTOME RNA POL I PROMOTER OPENING	49	-0.50684	-1.99001	0	0.007043

Supplementary Table S6.2 – Top 50 upstream regulators from Ingenuity Pathway Analysis, based on genes differentially expressed after 7 days of knee immobilization. Ranking based on p-value of overlap.

Upstream Regulator	Expr Log Ratio	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
ID2	-0.614	transcription regulator		0.302	1.82E-04
ID3	-0.276	transcription regulator		-0.56	7.22E-04
Insulin		group		-1.502	2.16E-03
ALKBH5	-0.033	enzyme			2.19E-03
IFI16	-0.448	transcription regulator		0.81	2.80E-03
CBS/CBSL		enzyme		1	3.32E-03
dihydrotestosterone		chemical - endogenous mammalian		0.908	3.67E-03
SPIB		transcription regulator		0.378	5.06E-03
MAP2K5	0.247	kinase			5.07E-03
cobalt		chemical toxicant			6.95E-03
FSH		complex		-0.322	7.11E-03
adenosine dialdehyde		chemical reagent			7.72E-03
HDAC4	1.677	transcription regulator		0.861	8.01E-03
COL6A1	-0.395	other		-1.342	8.31E-03
mir-223		microrna		-0.118	9.04E-03
FOXO3		transcription regulator	Activated	2.084	1.17E-02
dactolisib		chemical drug		1.951	1.25E-02
2-amino-5-phosphonovaleric acid		chemical - other		1.437	1.27E-02
NRL		transcription regulator		0	1.58E-02
clofibrate		chemical drug		1.963	1.59E-02
C8		complex			1.66E-02
glemanserin		chemical drug			1.66E-02
WISP3		growth factor			1.66E-02
GH2		other			1.66E-02
NKX6-1		transcription regulator			1.66E-02
C9		other			1.66E-02
C7		other			1.66E-02
KLK5		peptidase			1.66E-02
dimaprit		chemical drug			1.66E-02
quetiapine		chemical drug			1.66E-02
FOXP3		transcription regulator			1.72E-02
LAMTOR5	-0.008	other			1.74E-02
curcuminoids		chemical drug			1.74E-02
WNT11		other		1.342	1.86E-02
AK1	-0.739	kinase			2.02E-02

NFATC2	-0.06	transcription regulator	0.664	2.10E-02
RRP1B		other		2.47E-02
olanzapine		chemical drug	-0.895	2.48E-02
histamine		chemical - endogenous mammalian	0.885	2.59E-02
deoxycholate		chemical - endogenous mammalian	-0.394	2.59E-02
10E,12Z-octadecadienoic acid		chemical - endogenous mammalian	-1.698	2.64E-02
2,3,4,7,8-pentachlorodibenzofuran		chemical toxicant	0.478	2.76E-02
thiazolidinedione		chemical drug	-1.131	3.03E-02
BCL11B		transcription regulator		3.16E-02
MAP2K7		kinase		3.42E-02
PIAS1	0.23	transcription regulator	-0.447	3.48E-02
mir-25		microrna	-0.882	3.48E-02
tetrachlorodibenzodioxin		chemical toxicant	1.412	3.59E-02
Cg		complex	0.246	3.59E-02

Supplementary Table S6.3 – Top 50 genesets enriched during bed rest. Ranking is based on absolute normalized enrichment score (NES). ES: Enrichment score. NES: Normalized enrichment score. NOM p-val: nominal p-value. FDR q-val: P-value adjusted using Benjamini-Hochberg False Discovery Rate.

Name	Size	ES	NES	NOM p-val	FDR q-val
REACTOME TCA CYCLE AND RESPIRATORY ELECTRON TRANSPORT	116	-0.72647	-3.38487	0	0
REACTOME RESPIRATORY ELECTRON TRANSPORT ATP SYNTHESIS BY CHEMIOSMOTIC COUPLING AND HEAT PRODUCTION BY UNCOUPLING PROTEINS	80	-0.73657	-3.23365	0	0
REACTOME RESPIRATORY ELECTRON TRANSPORT	64	-0.75913	-3.18785	0	0
KEGG PARKINSONS DISEASE	111	-0.64511	-3.03839	0	0
REACTOME SRP DEPENDENT COTRANSLATIONAL PROTEIN TARGETING TO MEMBRANE	94	0.623695	3.000525	0	0
KEGG LYSOSOME	119	0.586164	2.967007	0	0
KEGG OXIDATIVE PHOSPHORYLATION	115	-0.63241	-2.95886	0	0
REACTOME PEPTIDE CHAIN ELONGATION	73	0.616106	2.882654	0	0
KEGG RIBOSOME	76	0.602861	2.8164	0	0
REACTOME PYRUVATE METABOLISM AND CITRIC ACID TCA CYCLE	40	-0.71285	-2.72647	0	0
REACTOME NONSENSE MEDIATED DECAY ENHANCED BY THE EXON JUNCTION COMPLEX	94	0.54543	2.701495	0	0
KEGG LEISHMANIA INFECTION	66	0.590432	2.68391	0	0
REACTOME TRANSLATION	129	0.509645	2.642031	0	0
REACTOME 3 UTR MEDIATED TRANSLATIONAL REGULATION	91	0.533291	2.602906	0	0
REACTOME INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	89	0.537578	2.594616	0	0
REACTOME INFLUENZA LIFE CYCLE	122	0.510243	2.57867	0	0
KEGG ALZHEIMERS DISEASE	154	-0.50725	-2.49155	0	0
KEGG HUNTINGTONS DISEASE	169	-0.49907	-2.47711	0	0
PID CXCR4 PATHWAY	102	0.500613	2.459188	0	1.63E-04
PID INTEGRIN A4B1 PATHWAY	33	0.630807	2.458056	0	1.49E-04
KEGG CITRATE CYCLE TCA CYCLE	30	-0.69513	-2.4298	0	0
PID IL8 CXCR2 PATHWAY	33	0.640461	2.426467	0	1.38E-04
REACTOME GENERATION OF SECOND MESSENGER MOLECULES	26	0.665985	2.415338	0	1.28E-04
REACTOME GPVI MEDIATED ACTIVATION CASCADE	31	0.634178	2.393021	0	3.63E-04
REACTOME INTERFERON GAMMA SIGNALING	57	0.538127	2.389887	0	3.41E-04
REACTOME OLFACTORY SIGNALING PATHWAY	297	-0.45242	-2.38508	0	0
KEGG FC GAMMA R MEDIATED PHAGOCYTOSIS	92	0.490054	2.378169	0	3.21E-04
REACTOME CITRIC ACID CYCLE TCA CYCLE	19	-0.76068	-2.36828	0	0
PID PI3KCI PATHWAY	49	0.554782	2.326147	0	5.00E-04
REACTOME PHOSPHORYLATION OF CD3 AND TCR ZETA CHAINS	15	0.734603	2.325055	0	4.74E-04
REACTOME PYRUVATE METABOLISM	18	-0.74499	-2.31748	0	0

REACTOME MITOCHONDRIAL FATTY ACID BETA OXIDATION	14	-0.7961	-2.29614	0	0
PID IL8 CXCR1 PATHWAY	27	0.628558	2.28687	0	8.01E-04
PID INTEGRIN1 PATHWAY	66	0.501866	2.284304	0	7.63E-04
REACTOME METABOLISM OF MRNA	195	0.416486	2.275443	0	8.10E-04
REACTOME P75 NTR RECEPTOR MEDIATED SIGNALLING	79	0.477519	2.273325	0	7.75E-04
REACTOME ASPARAGINE N LINKED GLYCOSYLATION	80	0.480908	2.273279	0	7.42E-04
REACTOME SIGNALING BY RHO GTPASES	110	0.453769	2.272689	0	7.13E-04
KEGG PRIMARY IMMUNODEFICIENCY	35	0.563493	2.264446	0	6.85E-04
REACTOME CYTOKINE SIGNALING IN IMMUNE SYSTEM	256	0.398189	2.251344	0	8.60E-04
KEGG OLFACTORY TRANSDUCTION	365	-0.41234	-2.24977	0	3.58E-05
KEGG CARDIAC MUSCLE CONTRACTION	72	-0.52381	-2.24287	0	6.74E-05
REACTOME REGULATION OF PYRUVATE DEHYDROGENASE PDH COMPLEX	12	-0.82535	-2.23817	0	9.49E-05
BIOCARTA NKCELLS PATHWAY	20	0.668843	2.234647	0	8.92E-04
KEGG INTESTINAL IMMUNE NETWORK FOR IGA PRODUCTION	45	0.534629	2.233325	0	9.22E-04
REACTOME MHC CLASS II ANTIGEN PRESENTATION	88	0.463759	2.229342	0	9.52E-04
REACTOME INFLAMMASOMES	16	0.701081	2.227516	0	9.79E-04
SA B CELL RECEPTOR COMPLEXES	24	0.618634	2.224591	0	0.001006
PID FCER1 PATHWAY	62	0.488736	2.215382	0	0.001083
PID TCR PATHWAY	66	0.485929	2.214418	0	0.001051

Supplementary Table S6.4 – Top 50 upstream regulators from Ingenuity Pathway Analysis, based on genes differentially expressed after 7 days of bed rest.Ranking based on p-value of overlap.

Upstream Regulator	Expr Log Ratio	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
KDM5A	0.112	transcription regulator	Activated	3.671	4.96E-09
KLF15	-0.794	transcription regulator	Inhibited	-3.711	4.31E-08
rosiglitazone		chemical drug	Inhibited	-4.104	6.72E-08
RXRA	-0.292	ligand-dependent nuclear receptor		-1.854	8.75E-08
PPARA	-0.694	ligand-dependent nuclear receptor	Inhibited	-3.315	1.18E-07
mono-(2-ethylhexyl) phthalate		chemical toxicant	Inhibited	-4.845	2.47E-07
TNF		cytokine	Activated	2.567	3.35E-07
PPARGC1A	-0.681	transcription regulator	Inhibited	-3.496	2.37E-06
INSR	-0.197	kinase	Inhibited	-4.265	3.00E-06
MAP4K4	0.458	kinase	Activated	4.375	5.36E-06
PPARD		ligand-dependent nuclear receptor	Inhibited	-2.393	8.57E-06
LEP		growth factor		-0.645	1.24E-05
Esrra		transcription regulator	Inhibited	-2.579	1.53E-05
calcitriol		chemical drug		-0.042	1.81E-05
dexamethasone		chemical drug		-1.161	1.93E-05
RB1	0	transcription regulator	Inhibited	-3.318	2.11E-05
sirolimus		chemical drug	Inhibited	-2.753	2.20E-05
RICTOR	0.027	other	Activated	3.796	6.44E-05
alitretinoin		chemical drug		-0.927	6.46E-05
Rxr		group	Activated	2.213	6.89E-05
ESRRA		ligand-dependent nuclear receptor	Inhibited	-3.052	7.94E-05
LIPG		enzyme		0.816	1.05E-04
OMA1	-0.245	peptidase		-1.633	1.05E-04
cholesterol		chemical - endogenous mammalian		1.44	1.12E-04
LPL	-0.82	enzyme		-0.578	1.16E-04
NR3C1	-0.146	ligand-dependent nuclear receptor		1.328	1.80E-04
MYOCD		transcription regulator		-0.957	1.87E-04
PGR		ligand-dependent nuclear receptor		-0.688	1.98E-04
CREB1	0.052	transcription regulator		0.831	2.07E-04
gemfibrozil		chemical drug		-1.367	2.36E-04
HDAC5	-0.287	transcription regulator		0	2.75E-04
TP73		transcription regulator		0.161	2.92E-04

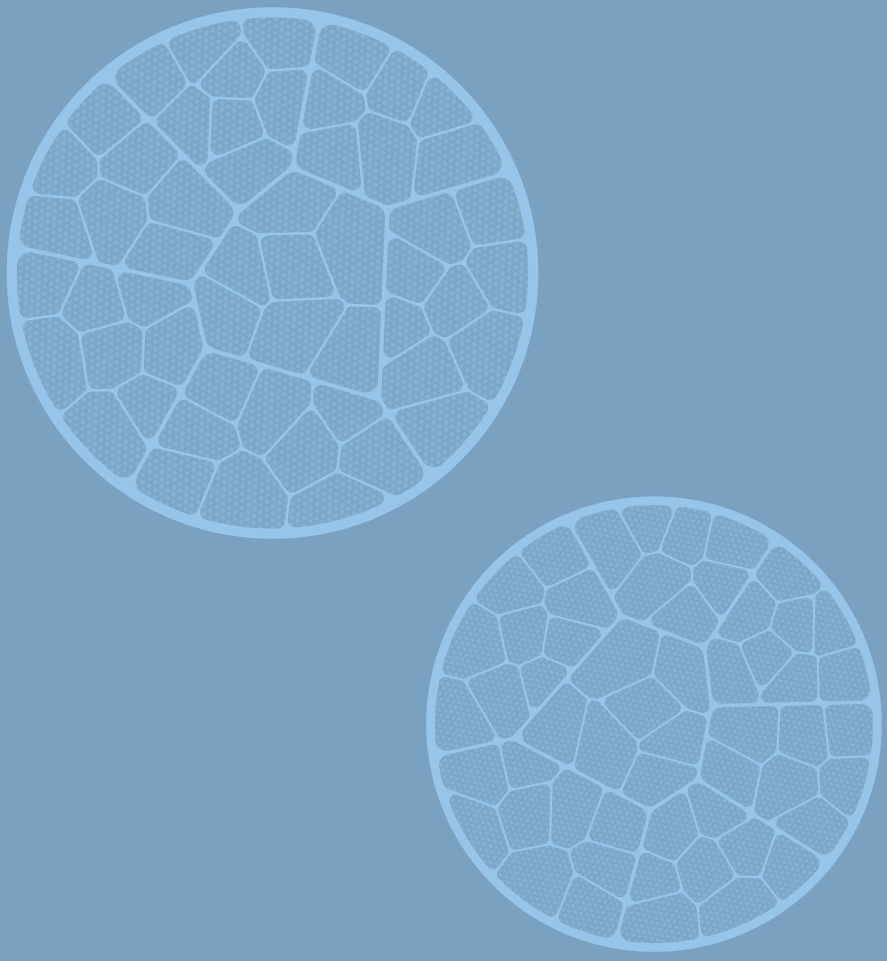
SPI1		transcription regulator	Activated	2.424	2.96E-04
troglitazone		chemical drug	Inhibited	-2.7	2.98E-04
Rar		group			3.10E-04
IL13		cytokine	Inhibited	-2.371	3.34E-04
CEBPA		transcription regulator		0.927	4.59E-04
NCOR-LXR-Oxysterol-RXR-9 cis RA		complex		0	4.74E-04
RXRG	-0.502	ligand-dependent nuclear receptor			4.74E-04
VDR		transcription regulator		0.231	4.75E-04
guanidinopropionic acid		chemical - endogenous non-mammalian	Inhibited	-3.29	4.94E-04
PIAS1	0.121	transcription regulator		-1.604	5.28E-04
KLF11		transcription regulator		-1.934	5.28E-04
IGF1R	-0.032	transmembrane receptor	Inhibited	-3.192	5.29E-04
Insulin		group		-0.498	5.61E-04
oleic acid		chemical - endogenous mammalian		-0.598	5.87E-04
NOTCH1		transcription regulator		-1.525	6.30E-04
FABP1		transporter		0.655	6.45E-04
galactosylceramide-alpha		chemical reagent		0.152	6.45E-04

Supplementary Table S6.5 – Top 50 genes across 8 datasets. Q-value is for the p-value derived using Fisher's method, corrected using Benjamini-Hochberg False Discovery Rate. Ranking based on Q-value.

Gene	ULLS (21 days)	Bed Rest (9 days)	Bed Rest (7 days)	KI (7 days)	Prot. Def. (7 days)	KI (2 days)	ULLS (2 days)	ULLS (1 day)	Q-value
CHRNA1	***0.000***	***0.005***	***0.000***	0.023	***0.000***	***0.005***	***0.003***	***0.002***	0.000
COL4A3	***0.001***	***0.005***	0.619	***0.001***	0.841	***0.000***	***0.000***	***0.004***	0.000
CALM1	***0.002***	***0.001***	***0.001***	***0.000***	0.756	***0.002***	0.033	0.026	0.000
COL4A4	***0.006***	***0.001***	0.776	***0.004***	0.775	0.042	***0.000***	***0.000***	0.000
PAQR9	***0.000***	***0.000***	***0.000***	0.716		0.778	***0.002***	***0.000***	0.000
COQ10A	***0.000***	***0.000***	***0.005***	***0.000***		0.068	0.111	***0.005***	0.000
COQ3	***0.004***	***0.000***	***0.008***	0.070	***0.002***	0.237	0.030	***0.001***	0.000
LSMEM2	***0.008***	***0.000***	***0.003***	***0.006***		***0.001***	0.130	0.015	0.000
ALPK3	***0.001***	***0.006***	***0.001***	0.046	***0.003***	0.678	***0.002***	0.015	0.000
GLRX	***0.008***	***0.001***	***0.001***	***0.001***	0.656	0.075	0.045	***0.006***	0.000
TSPAN8	0.241	***0.008***	0.876	0.141	***0.001***	***0.000***	***0.001***	***0.001***	0.000
MEGF10	***0.002***	***0.002***	***0.001***	***0.002***		***0.003***	0.076	0.579	0.000
RASL12	***0.005***	***0.005***	***0.001***	***0.002***	0.127	0.693	0.020	***0.003***	0.000
CNNM4	***0.000***	***0.000***	***0.000***	0.095	***0.008***	0.027	0.108	0.019	0.000
CDC42EP3	***0.000***	***0.000***	***0.009***	***0.000***	0.011	0.040	0.023	0.256	0.000
TFRC	***0.000***	0.854	0.019	***0.000***	0.010	***0.002***	***0.000***	0.209	0.000
CHRND	***0.000***	***0.000***	***0.000***	0.366	0.095	0.062	0.301	***0.003***	0.000
XPO4	***0.000***	***0.001***	***0.000***	0.025	0.720	***0.008***	0.060	0.104	0.000
TWF2	***0.000***	***0.000***	***0.000***	0.317	0.050	***0.001***	0.338	0.080	0.000
SMCO1	***0.000***		***0.007***	***0.000***		0.053	0.011	***0.002***	0.000
ACSS1	***0.000***	***0.000***	***0.000***	0.656		0.527	0.074	***0.005***	0.000
SLC7A6	***0.008***	0.033	0.090	***0.000***	0.108	***0.000***	***0.010***	0.014	0.000
CA14	***0.000***	***0.000***	***0.010***	***0.008***	0.881	0.109	0.275	0.189	0.000
PPIF	***0.000***	***0.001***	***0.000***	***0.001***	0.079	0.020	0.553	0.786	0.000
SLC16A10	***0.007***	0.055	0.315	***0.001***	0.154	***0.002***	0.014	***0.000***	0.000
TP53INP2	***0.004***	***0.001***	0.012	0.170		0.344	***0.000***	***0.000***	0.000
RORA	***0.001***	***0.008***	0.344	***0.000***	0.120	0.036	***0.008***	0.025	0.000
LPL	***0.000***	***0.002***	***0.000***	0.823	0.042	***0.006***	0.576	0.190	0.000
DCXR	***0.001***	***0.000***	***0.000***	0.396	***0.001***	0.694	0.876	0.156	0.000
HINT2	***0.000***	***0.000***	***0.000***	0.199		0.188	0.802	***0.002***	0.000
ITGB1BP2	***0.000***	***0.001***	0.283	***0.003***	0.016	0.043	0.199	***0.005***	0.000
FAM210A	***0.005***	***0.001***	***0.003***	0.036		0.023	***0.008***	0.034	0.000
KLHL40	***0.002***	***0.002***	0.321	***0.001***		***0.000***	0.117	0.354	0.000
C1orf54	0.271	0.734	0.051	0.032	***0.004***	***0.009***	***0.000***	***0.007***	0.000
TYRP1	***0.005***	***0.000***	***0.000***	0.044	0.330	0.225	0.058	***0.010***	0.000
ANKRD12	0.118	0.035	0.145	***0.000***	0.979	***0.010***	***0.007***	***0.000***	0.000

ZFC3H1	0.127	0.078	0.018	***0.009***	0.802	***0.000***	***0.001***	***0.004***	0.000
MYADML2	***0.002***		***0.001***	***0.004***		***0.003***	0.079	0.038	0.000
GOT1	***0.002***	***0.000***	***0.002***	***0.006***	0.247	0.191	0.261	0.109	0.000
PERM1	***0.001***	***0.006***	***0.003***	0.061		0.283	0.010	***0.004***	0.000
BCO2	***0.002***	***0.002***	***0.002***	0.058		0.947	0.033	***0.001***	0.000
DHTKD1	***0.001***	***0.001***	***0.003***	0.185	0.020	***0.004***	0.069	0.772	0.000
MRPL12	***0.002***	***0.001***	***0.003***	***0.001***	0.062	0.131	0.421	0.092	0.000
PFKFB3	0.016	0.067	0.274	***0.002***	0.108	***0.002***	***0.008***	***0.002***	0.000
CCDC39	***0.002***	***0.008***	***0.000***	***0.007***		0.407	0.078	0.208	0.000
PPP1R1C	***0.005***	***0.008***	***0.000***	0.531		0.036	0.021	***0.005***	0.000
DLST	***0.005***	***0.001***	***0.003***	0.208	0.107	0.139	***0.005***	0.014	0.000
TACO1	***0.002***	***0.002***	***0.006***	0.101	0.081	0.088	0.132	***0.003***	0.000
MRPL34	***0.010***	***0.003***	0.012	0.220	***0.004***	***0.007***	0.113	0.042	0.000
COL1A1	***0.001***	0.064	***0.009***	0.327	***0.004***	***0.002***	0.015	0.644	0.000

Summary



Prolonged lifespan and decreased fertility will lead to an increased proportion of older adults in the world population (population aging). An important strategy to deal with population aging has been to promote healthy aging; not only to prevent mounting health care costs, but also to maintain independence and quality of life of older populations for as long as possible. Close to the opposite of the healthy aging is frailty. A major component of (physical) frailty is sarcopenia: age-related loss of muscle mass. Decreased muscle size and strength has been associated with a wide variety of negative health outcomes, including increased risk of hospitalization, physical disability and even death. Therefore, maintaining muscle size and strength is very important for healthy aging. Nutrition and physical activity are possible strategies to maintain or even improve muscle function with age.

The effect of nutrition, age, frailty and physical activity on the function of skeletal muscle is complex. A better understanding of the molecular mechanisms involved can provide new insights in potential strategies to maintain muscle function over the life course. This thesis aims to investigate these mechanisms and processes that underlie the effects of age, frailty and physical activity by leveraging the sensitivity and comprehensiveness of transcriptomics and metabolomics.

Chapter 2 and 3 describe the effects of age, frailty and resistance-type exercise training on the skeletal muscle transcriptome and metabolome. Both the transcriptome and metabolome show significant differences between frail and healthy older adults. These differences are similar to the differneces between healthy young men and healthy older adults, suggesting that frailty presents itself as a more pronounced form of aging, somewhat independent of chronological age. These age and frailty related differences in the transcriptome are partially reversed by resistance-type exercise training, in accordance with the observed improvement in muscle strength. Regression analysis revealed that the protocadherin gamma gene cluster may be important to skeletal muscle function. Protocadherin gamma is involved in axon guidance and may be upregulated due to the denervation-reinnervation cycles observed in skeletal muscle of older individuals. The metabolome suggested that resistance-type exercise training led to a decrease in branched-chain amino acid oxidation, as shown by a decrease in amino acid derived carnitines. Lastly, the blood metabolome showed little agreement with the metabolome in skeletal muscle, indicating that blood is a poor read-out of muscle metabolism.

We assessed the effect of knee immobilization with creatine supplementation or placebo on the skeletal muscle transcriptome and metabolome in chapter 4. Knee immobilization caused muscle mass loss and strength loss in all participants, with no differences between creatine and placebo groups. Knee immobilization appeared to induce the HDAC4-myogenin axis, which is primarily associated with denervation and motor neuron diseases. The metabolome showed changes consistent with the decreased expression of energy metabolism genes. While acyl-carnitine levels tended to decrease with knee immobilization, one branched-chain amino acid-derived acyl carnitine was increased after knee immobilization, suggesting increased amino acid oxidation.

Vitamin D deficiency is common among older adults and has been linked to muscle weakness. Vitamin D supplementation has been proposed as a strategy to improve muscle function among older populations. In chapter 5, supplementation with vitamin D (calcifediol, 25(OH)D) is investigated as nutritional strategy to improve muscle function among frail older adults. However, we observed no effect of vitamin D on the muscle transcriptome. These findings indicate the effects of vitamin D supplementation on skeletal muscle may be either absent, weak, or limited to a small subset of muscle cells.

Transcriptomic changes due to different forms of muscle disuse are compared in chapter 6 (primarily knee immobilization and bed rest). The goal was to determine the similarities and differences among various causes of muscle atrophy in humans (primarily muscle disuse). Both knee immobilization and bed rest led to significant changes in the muscle transcriptome. However, the overlap in significantly changed genes was relatively small. Knee immobilization was characterized by ubiquitin-mediated proteolysis and induction of the HDAC4/Myogenin axis, whereas bed rest revealed increased expression of genes of the immune system and increased expression of lysosomal genes. Knee immobilization showed the highest similarity with age and frailty-related transcriptomic changes. This finding suggests that knee immobilization may be the most suitable form of disuse atrophy to assess the effectiveness of strategies to prevent age-related muscle loss in humans.

The transcriptome and metabolome are incredibly useful tools in describing the wide array of biological systems within skeletal muscle. These systems can be modulated using physical activity (or lack thereof) as well as nutrition. This thesis describes some of these processes and highlights several unexplored genes and metabolites that may be important for maintaining or even optimizing muscle function. In the future, it may be possible to optimize both exercise and nutrition for each individual using these techniques; or even better, cheaper and less invasive alternatives.

Acknowledgements

Het zit erop. Ochtenden, middagen en avonden zwoegen over talloze tabellen, grafieken, R scripts, presentaties en teksten. Te lang onder de douche staan om over problemen en oplossingen na te denken. Creatieve en minder creatieve ingevingen na een zware benentraining in de sportschool. Maar zonder steun, hulp en inspiratie van anderen was het afronden van mijn thesis helemaal niet gelukt. Daarom wil ik iedereen bedanken die heeft bijgedragen aan mijn onderzoek.

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PubMed alerts ☺ **Ineke**, bedankt voor het nemen van alle spierbiopten en de interessante gesprekken.

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About the author

Biography

Roland Willem Jan Hangelbroek was born without a beard on Friday the 14th of February 1986 in Rolde, Drenthe. Before his first birthday, his parents, Willem Hangelbroek and Sjoukje Hangelbroek-Sibie, moved Roland and his sister Femke to Leeuwarden. His innate curiosity was encouraged from an early age in the Hangelbroek household. Roland was taught to value knowledge and to pursue his many interests. After the purchase of a computer, most of Roland's focus became digital, as he taught himself programming, web design and 3D graphics.

At school, still beardless, Roland soon realized that by putting in the minimal effort on the material he was obligated to learn, he would have the maximum amount of time to spend on things that he considered actually interesting. At 17, first stubble appearing on his chin, Roland discovered weight lifting, a sport that piqued his interest in the human body and nutrition and that made him decide to study Life Science & Technology at the University of Groningen. After a year in Groningen, Roland moved to Wageningen in 2006 to study Voeding & Gezondheid, a Bachelor course that was more catered to his increased interest in nutrition. The highlight of his bachelor was his literature review of Fat Loss Supplements, which introduced Roland to the joy scientific writing.

In 2010, his beard becoming a more frequent presence on his cheeks, he chose to continue his studies in Wageningen with a master in Nutrition and Health with the specialization, Molecular Nutrition and Toxicology. He also broadened his horizon by following a minor in Marketing & Management and qualifying as a fitness instructor. In 2013 Roland received the Diploma in Sports Nutrition from the International Olympic Committee, a two year course that he successfully completed alongside his studies in Wageningen.

Triggered by the social relevance of research into nutrition and muscle health, Roland started his PhD on muscle health and nutrigenomics. For the first time, Roland's beard had become a permanent feature. During his PhD he was able to work on a subject with tangible use in society that interested him and allowed him to further develop skills he had honed in his free time. A combination that he found both motivating and captivating.

After his PhD Roland started work as data scientist at Vitens. He is continuing to learn and develop, while working on new big data puzzles. And his beard? His beard will continue adorn his visage.

LIST OF PUBLICATIONS

Expected articles

Hangelbroek, R.W.J., Backx, E.M.P., Kersten, S., Van Duynhoven, J.P.M., Verdijk, L.B., Van Loon, L.J.C, De Groot, L.C.P.G.M., Boekschoten, M.V. Creatine supplementation in young healthy men attenuates changes in skeletal muscle transcriptome during knee immobilization *(in preparation)*

Hangelbroek, R.W.J., Knuiman, P., Tieland, M., De Groot L.C.P.G.M., Attenuated strength gains during prolonged resistance exercise training in pre-frail older adults with high inflammatory status *(accepted with revisions)*

Hangelbroek, R.W.J., Vaes, A.M.M., Kersten, S., Boekschoten, M.V., Verdijk, L.V., Van Loon, L.J.C, De Groot, L.C.P.G.M., No effect of calcifediol supplementation on skeletal muscle transcriptome in vitamin D deficient frail older adults *(in preparation)*

Hangelbroek, R.W.J., Dirks, M.L., Backx, E.M.P., Verdijk, L.V., Van Loon, L.J.C., De Groot, L.C.P.G.M., Kersten, S., Boekschoten, M.V. The transcriptomic signature of muscle atrophy after knee immobilisation most closely resembles the transcriptomic signatures of frailty and age *(in preparation)*

Fazelzadeh, P., **Hangelbroek, R.W.J.**, Joris, P., Mensink, R., Esser, D., Afman, L., Hankemeier, T., Jacobs, D., Mihaleva, V., Kersten, S., Van Duynhoven, J., Boekschoten, M.V. Weight loss moderately affects the mixed meal challenge response of the plasma metabolome and transcriptome of peripheral blood mononuclear cells in obese subjects *(in preparation)*

Grootswagers, P., Vaes, A.M.M., **Hangelbroek, R.W.J.**, Tieland, M., Van Loon, L.J.C., De Groot, L.C.P.G.M. The reliability and validity of isometric lower extremity strength assessed by hand-held dynamometer in a population of community dwelling seniors *(in preparation)*

Knuiman, P., Hopman, M.T.E., **Hangelbroek, R.W.J.**, Mensink, M. Myokine responses to resistance exercise with different nutrient availability on a combined exercise day in trained healthy males *(in preparation)*

Michielsen, C.J.R., **Hangelbroek, R.W.J.**, Feskens, E.J.M., Afman, L.A.. Disentangling the effects of monounsaturated fat from other components of a Mediterranean diet on serum metabolite profiles: a randomized fully controlled trial in healthy subjects at risk of the metabolic syndrome *(in preparation)*

Published articles

Backx, E. M., **Hangelbroek, R.W.J.**, Snijders, T., Verscheijden, M. L., Verdijk, L. B., de Groot, L. C., & van Loon, L. J. 2017. Creatine Loading Does Not Preserve Muscle Mass or Strength During Leg Immobilization in Healthy, Young Males: A Randomized Controlled Trial. Sports Medicine, 1-11.

Wardenaar, F.C., Ceelen, I.J., Van Dijk, J.W., **Hangelbroek, R.W.J.**, Van Roy, L., Van der Pouw, B., De Vries, J.H., Mensink, M. and Witkamp, R.F., 2016. Nutritional Supplement Use by Dutch Elite and Sub-Elite Athletes: Does Receiving Dietary Counselling Make a Difference? International Journal of Sport Nutrition and Exercise Metabolism

Hangelbroek, R.W.J., Fazelzadeh, P., Tieland, M., Boekschoten, M.V., Hooiveld, G.J., Duynhoven, J.P., Timmons, J.A., Verdijk, L.B., Groot, L.C.P.G.M., Loon, L.J. and Müller, M., 2016. Expression of protocadherin gamma in skeletal muscle tissue is associated with age and muscle weakness. Journal of cachexia, sarcopenia and muscle, 7(5), pp.604–614.

Fazelzadeh, P., **Hangelbroek, R.W.J**., Tieland, M., de Groot, L.C., Verdijk, L.B., van Loon, L.J., Smilde, A.K., Alves, R.D., Vervoort, J., Müller, M. and van Duynhoven, J.P., 2016. The muscle metabolome differs between healthy and frail older adults. Journal of proteome research, 15(2), pp.499-509.

Catoire, M., Mensink, M., Boekschoten, M.V., **Hangelbroek, R.W.J**., Müller, M., Schrauwen, P. and Kersten, S., 2012. Pronounced effects of acute endurance exercise on gene expression in resting and exercising human skeletal muscle. PLoS One, 7(11), p.e51066.

Overview of completed training activities

Discipline specific activities

- Symposium 'Hoe verkoop je de simpele boodschap van gezond eten?' (Den Haag, 2013)
- Dutch Nutritional Science Days (Deurne, 2013)
- TIFN Annual Meeting 2013 (Amsterdam, 2013)
- Advanced visualisation, integration and biological interpretation of -omics data (Wageningen, 2014)
- Signal proteins related to diet and exercise (Oslo, 2014)
- TIFN Annual Meeting 2014 (Utrecht, 2014)
- Nugoweek (Naples, 2014)
- STATegra summer school: omics and data integration (Benicassim, 2015)
- TIFN Annual Meeting 2015 (Vlaardingen, 2015)
- TIFN Annual Meeting 2016 (Wageningen, 2016)
- Nugoweek (Copenhagen, 2016)

General courses

- VLAG PhD week (Baarlo, 2012)
- MBTI workshop ('s Hertogenbosch, 2013)
- Intelectual Property (Wageningen, 2013)
- Reviewing a scientific paper (Wageningen, 2015)
- Scientific Publishing (Wageningen, 2016)
- Philosophy and Ethics of Food Science and Technology (Wageningen, 2017)

Optional activities

- Preparation of research protocol (Wageningen, 2013)
- PhD study tour (US East Coast, 2015)
- Weekly NMG group meetings (Wageningen, 2012-2017)
- Muscle Meetings (Wageningen, 2013-2017)
- TIFN Expert Meetings (Wageningen, 2012-2017)

Colophon

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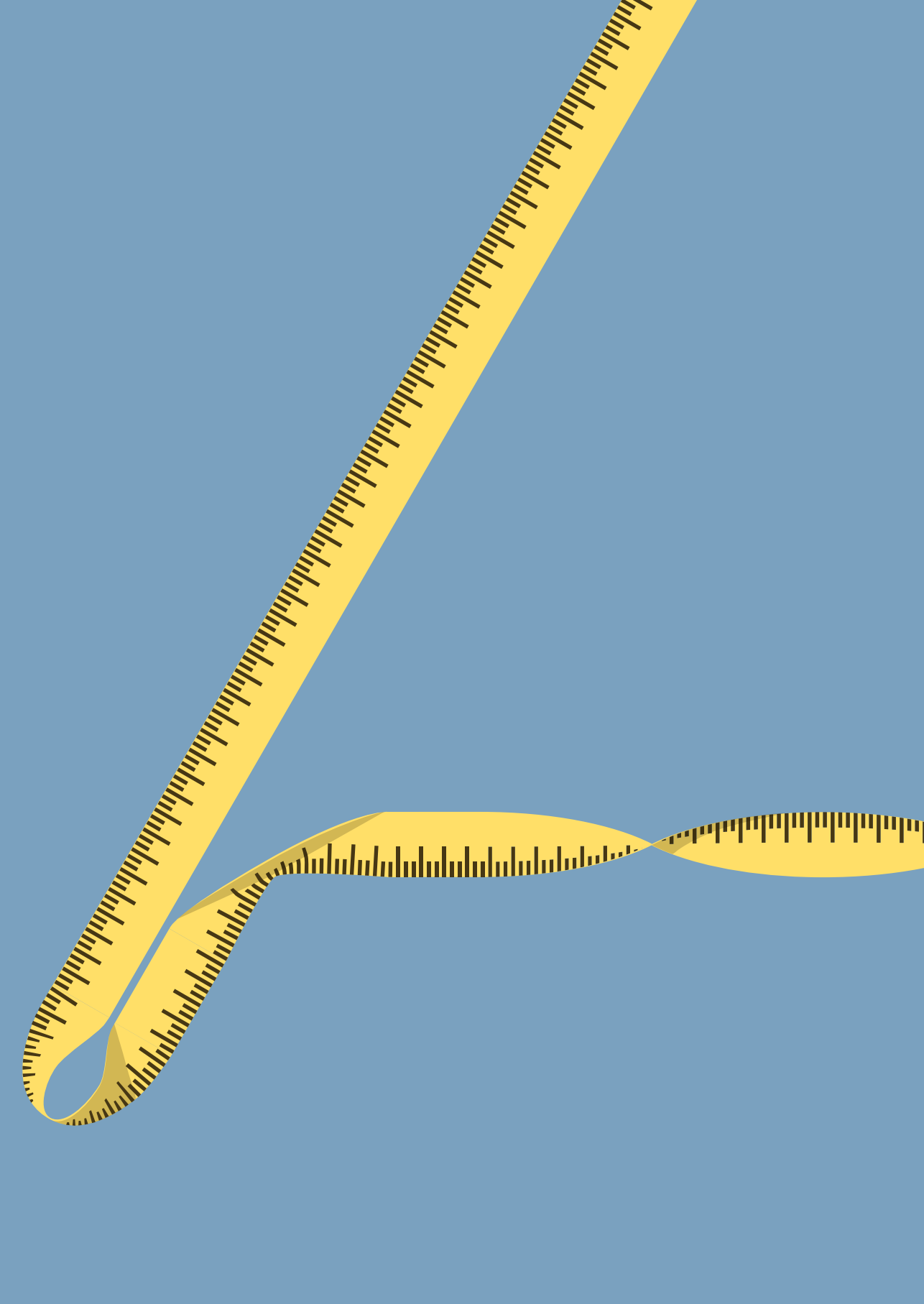

Propositions

1. Steady state gene expression can be used as a read-out of muscle function
   (this thesis)

2. Leg immobilization and bed rest have distinct effects on the skeletal muscle transcriptome
   (this thesis)

3. Psychedelic drugs have therapeutic potential in the treatment of mental disorders

4. Self-control is not a finite mental resource

5. Computer programming should be a required subject in high school

6. Contrary to popular belief, cats prefer social interaction over food

Propositions belonging to the thesis, entitled:

Molecular assessment of muscle health and function. The effect of age, nutrition and physical activity on the human muscle transcriptome and metabolome

Roland W.J. Hangelbroek

Wageningen, 15 December 2017