Dairy protein, exercise and inflammatory markers in older adults

Gyrd Omholt Gjevestad

Dissertation for the degree of Philosophiae Doctor (PhD)

Department of Nutrition
Institute of Basic Medical Sciences
Faculty of Medicine
University of Oslo

2017

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo

ISBN 978-82-8377-046-9

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.

Acknowledgements

The work presented in this thesis has been carried out at the Department of Nutrition, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo. The work has been financially supported through the industrial Ph.D. scheme of The Research Council of Norway and TINE SA (grant number 225258/E.40).

The years spent on this work have been educational and interesting, sometimes a bit frustrating, but most of the time; a lot of fun. I`ve never regretted accepting this opportunity. I`ve gained a lot of knowledge during these years, making me more qualified for further work in the nutrition field. It has also made me more confident debating nutrition in general, but also nutrition research.

This work had not been possible to finish without assistance and valuable support from my supervisors, Kirsten Bjørklund Holven, Stine Marie Ulven and Anne Sofie Biong. They have closely followed my along the way and provided excellent guidance and support. I would also like to express my gratitude to TINE SA and Johanne Brendehaug, head of TINE R&D, who gave me the opportunity to do this work.

Thanks to Håvard Hamarsland and Truls Raastad for collecting muscle biopsies and letting us harvest blood samples from their intervention studies, to Inger Ottestad, Lena Leder, Ingunn Narverud, Jacob J. Christiansen, Patrik Hansson, Sunniva V. Larsen, Linn Kristin L. Øyri and Håvard for valuable input and discussions throw-out these years, to Marit Sandvik and Ingunn for valuable assistance in the lab and to all my co-authors for valuable comments and contributions to the papers. A special thanks to Inger, Jacob, Håvard, Truls, Anne Sofie, Stine and Kirsten for carefully reading through my thesis as it was drafted, always providing thorough and reflected inputs to the manuscript.

Lastly, I am grateful to my family, especially my husband, who has taken full responsibility for organizing the family while I`ve spend late afternoons collecting samples, running time-consuming analysis in the lab or writing papers.

Oslo, February 2017

Gyrd Omholt Gjevestad

Table of contents

Lists of papers

Paper I

Inger Ottestad, Amund Tjellaug Løvstad, Gyrd Omholt Gjevestad, Håvard Hamarsland, Jūratė Šaltytė Benth, Lene Frost Andersen, Asta Bye, Anne Sofie Biong, Kjetil Retterstøl, Per Ole Iversen, Truls Raastad, Stine M Ulven, Kirsten B Holven. *Intake of a protein-enriched milk and effects on muscle mass and strength. A 12-week randomized placebo controlled trial among community-dwelling older adults*. J Nutr Health Aging (2016). doi:10.1007/s12603-016-0856-1

Paper II

Gyrd O. Gjevestad, Inger Ottestad, Anne Sofie Biong, Per Ole Iversen, Kjetil Retterstøl, Truls Raastad, Bjørn S. Skålhegg, Stine M. Ulven and Kirsten B. Holven. *Consumption of protein-enriched milk has minor effects on inflammation in older adults - a 12-week double-blind randomized controlled trial.* In press, Mechanisms of Ageing and Development. http://dx.doi.org/10.1016/j.mad.2017.01.011

Paper III

Gyrd O. Gjevestad, Håvard Hamarsland, Truls Raastad, Inger Ottestad, Jacob J. Christensen, Kristin Eckardt, Christian A. Drevon, Anne S. Biong, Stine M. Ulven and Kirsten B. Holven. *Gene expression is differentially regulated in skeletal muscle and circulating immune cells in response to an acute bout of high-load strength exercise.* In press, Genes & Nutrition.

Paper IV

Gyrd O. Gjevestad, Håvard Hamarsland, Truls Raastad, Jacob J. Christensen, Anne S. Biong, Stine M. Ulven and Kirsten B. Holven. *Eleven weeks of strength training decreased inflammatory markers in older subjects independent of protein supplement type; a randomized controlled trail*. Submitted manuscript.

Abbreviations

α-LA	alfa-lactalbumin
β-LB	beta-lactoglobulin
BCAA	branched chain amino acids
CCL	chemokine (C-C motif) ligand
CRP	C-reactive protein
CVD	cardiovascular disease
CXCL	chemokine (C-X-C motif) ligand
DPP4	dipeptidyl-peptidase 4
EAA	Essential amino acid
HIF1A	hypoxia-inducible factor 1-alpha
HiOA	Oslo and Akershus University College of Applied Sciences
IL	interleukin
INFG	interferon gamma
JNK	c-Jun N-terminal kinase
mRNA	messenger ribonucleic acid
mTORC1	mammalian target of rapamycin complex 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	The Norwegian School of Sports Sciences
NR1H3	nuclear receptor subfamily, group H, member 3
NR4A2	nuclear receptor subfamily 4, group A, member 2
NR4A3	nuclear receptor subfamily 4, group A, member 3
UiO	University of Oslo
PBMC	peripheral blood mononuclear cells
PPARGC1A	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPARGC1B	peroxisome proliferator-activated receptor gamma coactivator 1-beta
RT-qPCR	real-time quantitative polymerase chain reaction
TLDA	Taqman low-density array
TLR	toll-like receptor
TNF	tumor necrosis factor alpha
TNFRSF1A	tumor necrosis factor receptor superfamily member 1A
WPC	whey protein concentrate
WPI	whey protein isolate

1 Introduction

The role of nutrients, dietary patterns and physical activity in determining health are well established. Stimulating a healthy eating pattern and increasing the level of physical activity are important measurements to improve public health [1, 2]. While nutritional research in the first half of the 20th century mainly focused on providing sufficient amounts of nutrients to avoid deficiencies, the emphasis of modern nutrition research is given to interventions reducing the risk of developing chronic diseases, such as cardiovascular diseases (CVD), cancers and type 2 diabetes, and to promote healthy aging [3, 4]. Chronic diseases are the major cause of death in almost all countries, giving the prevention of chronic diseases high priority worldwide. The incidence of chronic diseases increases with increasing age [5]. The older population is estimated to increase substantially the next years, making it important to promote healthy aging for the purpose of increasing quality of life and enabling older adults to remain living at home as long as possible. There is a substantial amount of knowledge about the effects of nutrients, dietary patterns and physical activity on health, but the optimal levels to promote health, and the molecular mechanisms behind these effects are still largely unknown. Extensive focus is therefore given to research aiming at understanding how diet and physical activity optimally affect health and to reveal possible mechanisms behind such effects [6].

1.1 Dietary protein and health

Dietary proteins provide the human body with amino acids, which may serve as substrates for protein synthesis, precursors for enzymes and cellular structures or substrates for energy metabolism [7, 8]. Amino acids are essential for growth, development, reproduction, lactation, and survival of the organism [9]. They are classified as either essential, conditionally essential or non-essential, as shown in table 1, depending on the body`s ability to synthesize the amino acid at a rate sufficient to meet the requirements to maintain optimal growth [8]. Further, amino acids are able to modify gene expression at the level of transcription, mRNA stability and translation [7, 10-12].

Table 1. Classification of amino acids in humans [8].

Essential amino acids	**Conditionally essential amino acids**	**Nonessential amino acids**
Histidine	Arginine	Alanine
Isoleucine	Cysteine	Aspartate
Lecine	Glutamine	Asparagine
Lysine	Glycine	Glutamate
Methinonin	Proline	Serine
Phenylalanine	Tyrosine	
Threonine		
Trypotphan		
Valine		

Unlike the metabolism of carbohydrate and fat, there are no indispensable amino acid stores, making it important for mammals to regulate amino acid homeostasis precisely [7]. The pool of free amino acids in the body is small and is determined by the balance between input (intake from dietary proteins, and *de novo* synthesis) and removal (protein degradation). This balance can be challenged during protein malnutrition, imbalanced diets or various forms of stress, such as trauma and sepsis [10]. A crucial factor for promoting optimal health is to preserve protein homeostasis [13].

Skeletal muscle is the major reservoir of body protein [14] and contributes significantly to the overall energy and protein metabolism [15, 16]. Most amino acids are metabolized in the liver, but the branched chain amino acids (BCAA), leucine, isoleucine and valine, largely escape first-pass hepatic catabolism and are directly transported into the blood stream [15]. The plasma concentration of BCAA will therefore increase more than the other amino acids after a meal [15]. Skeletal muscle is the main site for metabolism of these amino acids. Essential amino acids (EAAs) enter skeletal muscle as substrates to promote protein synthesis [17], but they may also function as independent signals for the initiation of protein synthesis [18-20] and possibly also muscle protein breakdown [21, 22]. An inadequate amount of dietary protein may lead to catabolism of structural and functional proteins and decreased immune response, ultimately leading to reduced physiological function [23]. Early disruption of muscle biology has been observed after only two weeks of inadequate (0.5 g/kg/day) or marginal (0.75

g/kg/day) protein intakes in both young and older adults [23], showing that it is important to ensure an adequate protein intake across all parts of the population.

The estimated average requirement for dietary protein in adults (above 18 yrs) is 0.66 g protein/kg body weight [24] and the recommended daily intake is 0.8 g protein/kg body weight for adults [25]. In the Nordic countries, the recommended daily protein intake in older adults (> 65 years) was raised to 1.1-1.3 g protein/kg body weight in the Nordic nutrition recommendations from 2014 [25]. The increased recommendation for protein in older adults was made to counteract losses and maintain muscle mass and strength in aged subjects [25] as research suggest that older subjects have a greater need for protein [26, 27] and that muscle protein synthesis in older adults is less efficient than in younger adults [28, 29], a phenomenon defined as anabolic resistance [30]. Others have suggested that the optimal amount of protein needed to maintain muscle function in older adults is even higher; up to 1.5-1.6 g protein/kg body weight/day [20, 31, 32]. Anabolic resistance may be caused by several factors, such as dysregulation of intracellular signaling, reduction in postprandial nutritional flow, chronic inflammation, greater retention of dietary amino acids by the gut and liver and reduced activity levels [33].

Most Norwegian adults (18-65 yrs) have a protein intake according to the recommendations [34], whereas the situation for older adults is less clear as few dietary surveys among healthy Norwegian adults ≥ 70 years, living at home, has been performed [35]. However, European data indicate that 5–10% of community-dwelling people ≥ 70 years are undernourished [36]. Another aspect of protein research is to define the optimal protein intake. To establish the level of optimal intake may be relevant in sports to improve performance and promote optimal recovery [37], in the general population to prevent development of disease [38], in dieting to promote satiety [39, 40], and during aging to maintain muscle mass and strength [41]. Loss of muscle mass and strength in older adults may ultimately lead to the development of sarcopenia, which again is a predictor of all-cause mortality [42]. To prevent the development of sarcopenia, several measurements have been suggested, among them increased protein intake [43, 44], the use of fast versus slowly absorbed proteins [45] and supplementation with leucine [46]. However, there has been some skepticism towards using high-protein diets, especially in older subjects, as some studies have indicated a potential negative effect on kidney function [47].

Beneficial effects of high protein diets on the underlying factors of chronic diseases have scarcely been investigated, but evidence indicates that high protein diets improve blood

pressure, high-density lipoprotein cholesterol and triglyceride levels when compared to diets high in carbohydrates [48, 49]. Long-term studies with high-protein diets are rare, but reduced levels of inflammatory markers were observed in obese women who followed a high protein diet for 6 months compared to subjects following a high carbohydrate diet [50]. The health outcome of high-protein diets may also depend upon the type of protein consumed as replacing animal protein with vegetable protein has been shown to lower the risk of developing type 2 diabetes, whereas a higher intake of low-fat dairy products have been shown to reduce the risk of type 2 diabetes compared to commonly consumed sources of animal protein [51].

To summarize, optimal protein intake is important for maintenance and increased muscle mass and muscle strength in young as well as in older adults. Further, optimal protein intake may be important for a short recovery period in young athletes. It is also important to recognize that different protein sources may affect certain health outcomes differently.

1.1.1 Dairy protein

Milk from ruminants is an important food component in the Norwegian diet, either directly or as a commodity for different dairy products, such as cheese, butter, sour crème and yoghurts [52]. A daily intake of low-fat dairy products is recommended as part of a healthy diet because milk and dairy products are good sources of protein, calcium, iodine and several B-vitamins [52]. Milk contains approximately 87 % water, 3.3 % protein, 4.0 % fat, 4.6 % lactose, 0.7 % mineral substances, 0.17 % organic acids and 0.15 % other substances, such as enzymes [53]. Further, milk protein consists of approximately 80 % casein and 20 % whey proteins, which again contain several smaller protein fractions, as illustrated in Figure 1 [54].

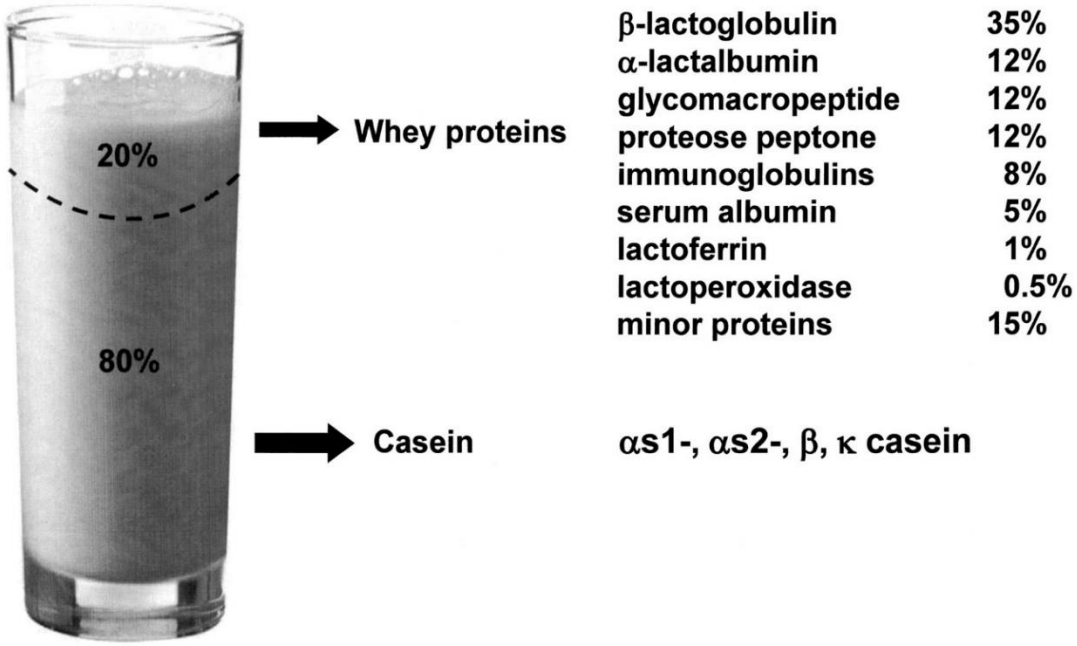


Figure 1. Milk protein consists of approximately 80% casein and 20 % whey, where whey protein consists of several smaller protein fractions as illustrated in percent contribution of the whey component. The composition of whey proteins may vary depending on production method. Casein further consists of αs1, αs2, β and κ casein. Reprint from Krissansen, *2007* [54], with permission from Taylor & Francis.

Casein is the main component of cheese making, leaving whey protein a waste product from cheese production [53]. Whey protein has traditionally been used as pet foods, but is nowadays recognized as a valuable nutritional source of human consumption. Whey protein is widely used in sports products and other foods aiming at fast absorption and rapid muscle growth [55, 56]. Common whey products are whey protein powders, such as whey protein concentrates (WPC) or whey protein isolates (WPI). WPC contains about 80 % protein, named WPC80, whereas WPI often contain 90% protein, named WPI90. Lactose is usually removed from WPI, whereas WPC often contains lactose. Advances in technology make it possible to produce whey products with different compositions, providing a wide range of products with different technological and nutritional properties on the market [57, 58].

Milk and dairy products may contain a substantial amount of fat, especially saturated fat, which has been shown to increase cholesterol and thereby the risk of cardiovascular vascular diseases (CVDs) [52]. However, studies also show that consuming dairy products, as part of a healthy diet, may promote satiety [59], reduce blood pressure [60] and promote insulin sensitivity [61],

suggesting that milk and dairy products are complex products with a wide range of nutrients and bioactive components potentially affecting other health parameters than cholesterol.

1.2 Chronic low-grade inflammation

The immune system plays an important role in providing protection to the body from infectious diseases and through wound healing. It initiates pathogen killing as well as tissue repair and helps to restore homeostasis [62]. Due to factors, such as chronic oxidative stress, increased age or other environmental factors, e.g. unhealthy diets or physical inactivity, an imbalance in the immune system may occur, potentially leading to chronic low-grade inflammation [63-66]. Chronic low-grade inflammation is characterized by constantly elevated levels of circulating inflammatory markers, such as interleukin (IL) 6, IL1β and tumor necrosis factor alpha (TNF), and is thought to play an important role in the pathogenesis of several chronic diseases, among them CVDs [67, 68], the metabolic syndrome [69, 70], type 2 diabetes [71-74] and obesity [75]. Cytokines include a broad group of molecules, such as interleukins and chemokines, and are important in cell signaling and communication [76]. They can mediate intercellular contact when bound to cell membranes or mediate communication between different cell types or tissues when secreted, acting either in an autocrine, paracrine or endocrine fashion [77]. Cytokines may be produced by different types of cells, including immune cells [78], adipocytes and skeletal muscle [79, 80].

1.2.1 Age-related chronic low-grade inflammation

Constantly elevated levels of cytokines (chronic low-grade inflammation) are often observed in older adults and may be referred to as inflammaging [66, 81]. Chronic low-grade inflammation is a robust predictor of disability and mortality, even in the absence of clinical disease [82, 83]. The etiology underlying inflammaging is not fully understood, but accumulative oxidative damage, increased visceral adiposity, reduced levels of sex hormones [82] and a dysregulations of the immune system, including failure in resolving inflammation, may play a role [84]. Different tissues (e.g. skeletal muscle), organs (e.g. liver) and systems (e.g. immune system) may contribute to the systemic chronic low-grade inflammation [85]. Further, an elevated inflammatory state can trigger or facilitate the onset of age-related diseases, such as sarcopenia [85-87], which is defined as a decline in muscle mass, muscle strength and functional performance [88]. An association between increased levels of CRP and IL6 with

reduced muscle mass and strength has been observed in several studies [89-92]. In addition, a strong relationship exists between muscle protein synthesis and circulating concentrations of several cytokines, such as TNF [93]. It has been hypothesized that TNF inhibit muscle protein synthesis by blunting the phosphorylation of mammalian target of rapamycin complex 1 (mTORC1) [94]. To reduce the level of chronic low-grade inflammation through changes in diet and increased physical activity levels are therefore recognized as important tools to promote healthy aging [82, 87].

1.2.2 Dairy protein and chronic low-grade inflammation

Components of the diet have the ability to modulate pathways involved in inflammation, transcription factors and inflammatory mediators [62]. Dietary components exerting beneficial effects on the immune system are fruits and vegetables, fish and whole grains, whereas the role of dietary carbohydrate and fat are more varying [75, 95], whereas less is known about potential effects of dietary protein on these markers [95]. Some epidemiological studies indicate that low-fat dairy products are able to exert anti-inflammatory effects by reducing the level of inflammatory markers [96]. However, the data are not conclusive [97, 98], and conclusions from randomized controlled trials have been conflicting [99]. Whey products have also been investigated for their potential effects on chronic low-grade inflammation. In short-term studies, whey protein has shown neutral [100, 101] but also anti-inflammatory properties [102, 103]. In long-term studies the results have been conflicting [104-106]. Milk and whey protein consist of several components, and some of them have been observed to inhibit inflammation [107-112] as illustrated in Figure 2.

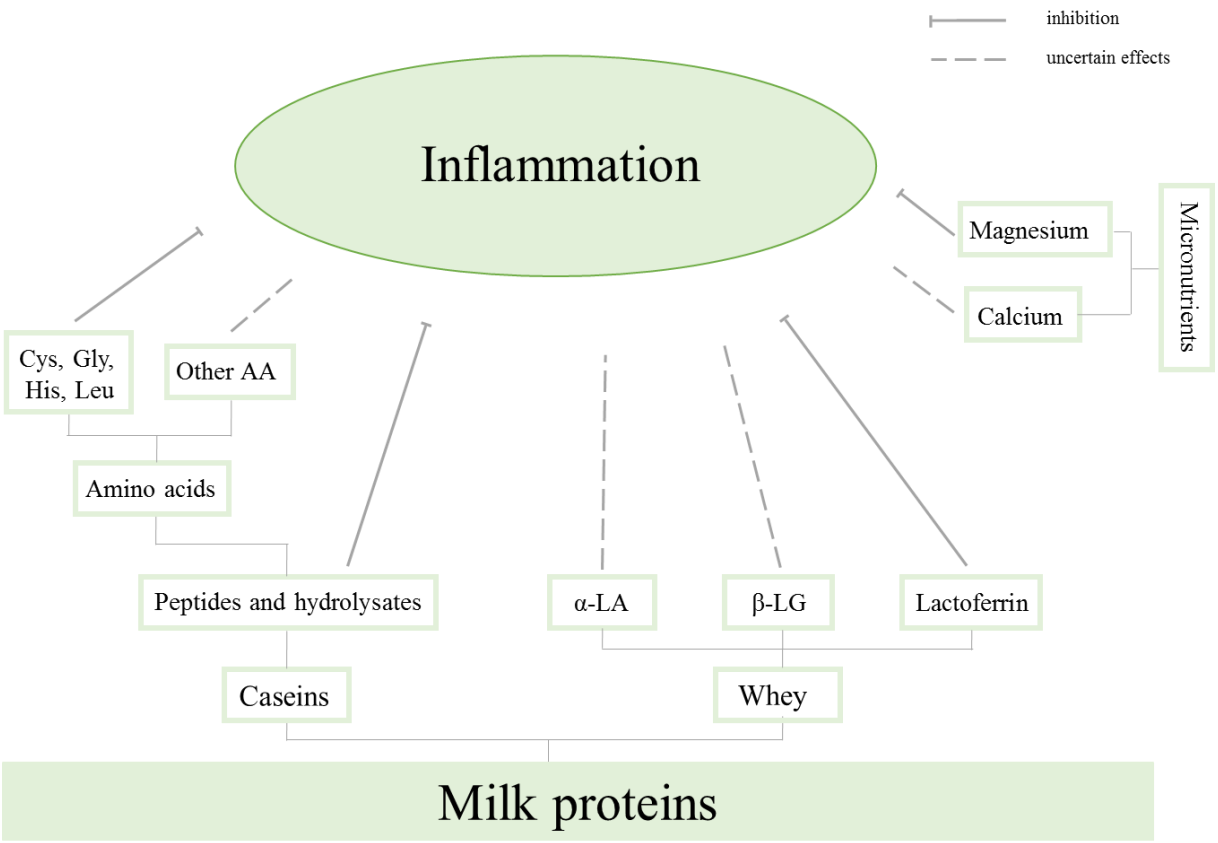


Figure 2. Components of milk proteins with potential effects on markers of inflammation. Solid lines represent known anti-inflammatory effects, while dotted lines indicate uncertain effects. Abbreviations used in figure; α-LA, alfa-lactalbumin; β-LB, beta-lactoglobulin; CLA, conjugated linoleic acids. Modified from Da Silva *et al*, 2015 [113], with permission from Wiley-VCH Verlag GmbH & Co. KGaA.

Possible mechanisms behind the potential anti-inflammatory effects of dairy proteins are, however, largely unknown, and may be part of complex interactions between dairy components, other nutrients and metabolic processes.

1.3 Aging and age-related loss of muscle mass

The Norwegian population is estimated to pass seven million people by 2060, doubling the population > 70 years towards 2060 [114]. Similar estimates are provided for the world wide increase of people > 60 years [115]. As the population grows, the incidence of chronic non-communicable diseases, including sarcopenia, is estimated to increase extensively, aggregating the societies economic burden. To prevent this development, promoting a healthy diet and

increasing physical activity levels are important measurements for the individual, but also for public health.

The aging process is multifactorial, and may be determined by a combination of genetic disposition and environmental factors [3], such as physical inactivity, malnutrition, obesity, increased inflammation and oxidative stress [31]. Further, inadequate protein intake [31, 116] and reduced ability to utilize available protein [14] may play a role. Aging is also associated with increased visceral adiposity [82], a decline in sex hormones [82], changes in muscle fiber composition [117] and in energy expenditure [118]. Further, aging is strongly associated with loss of muscle mass and muscle strength [82] and from the age of 50 yrs, muscle mass and muscle strength gradually decrease [119]. Age-related loss of muscle mass and muscle strength may ultimately lead to sarcopenia, which may impair functionality, reduce ability to manage activities of daily life [17, 120], reduce quality of life [121], increase morbidity and also mortality [26, 42, 122]. The prevalence of sarcopenia in Norway is not known, but numbers from other countries vary between 1-29 % for adults > 50 yrs living at home [123-126]. Preventing loss of muscle mass and strength are therefore important measurements to promote healthy aging. Current evidence suggests that protein supplementation may be a proper strategy [127] as higher intakes of dietary protein (e.g. 1.2 g/kg/day) may significantly decrease loss of muscle mass compared to lower intakes (e.g. 0.8 kg/g/day), illustrated in figure 3.

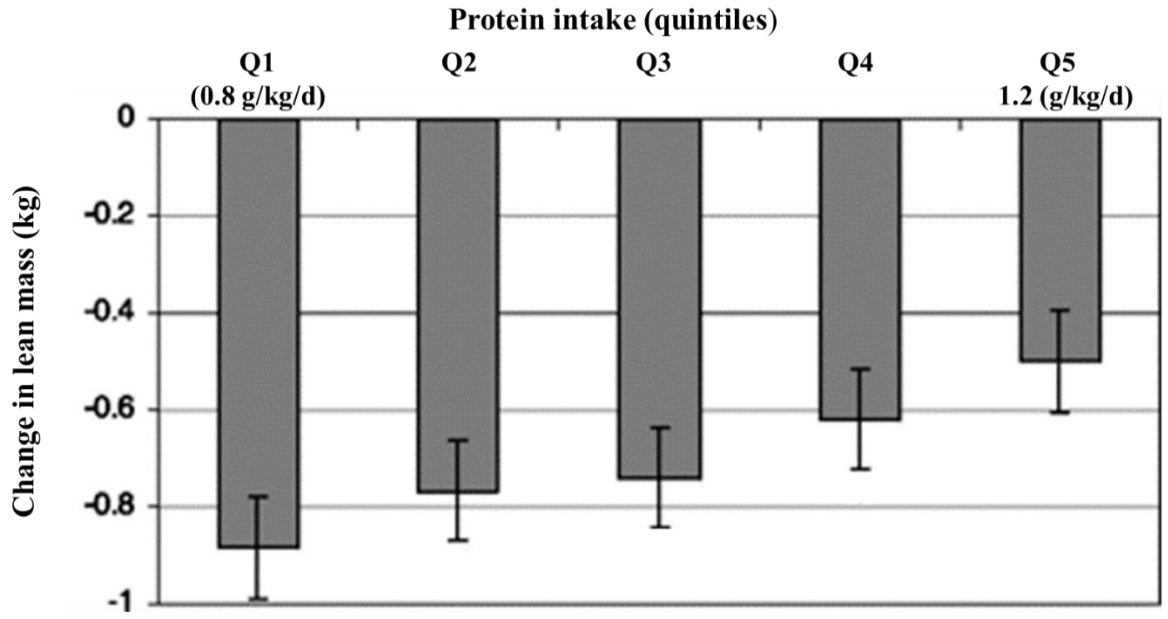


Figure 3. Subjects (70-79 yrs) in the highest protein quintile lost significantly less lean mass than those in the lowest protein quintile. Subjects were followed for three yrs. Modified from Houston *et al,* 2008 [128], with permission from American Society for Nutrition.

Further, EAAs, especially leucine [129], stimulate muscle protein synthesis [18], but the level required for an optimal stimulation of protein synthesis is still uncertain. Several studies report reduced muscle protein synthesis in older adults at protein intakes < 20 g protein/meal, while similar responses as in younger adults were observed at protein intakes > 20 g protein/meal [130-133]. Based on these results, it was suggested that older adults need higher amounts of EAAs, compared to younger adults, to stimulate muscle protein synthesis (anabolic resistance). The cause of this phenomenon is not known, but increased oxidative stress, inflammation, lower insulin sensitivity, decreased capacity of digestion and absorption of protein and amino acids and greater amino acid retention by splanchnic area may all play important roles [20]. Further, inactivity is suggested to be an important triggering factor in the development of anabolic resistance [134] as basal rates of muscle protein synthesis seems to differ minimally between young and older adults [20, 135]. If the distribution of protein throughout the day, compared to a single dose, is important in maintaining muscle mass in older adults have also been a question of debate and the results from clinical trial have been conflicting [136, 137].

Elements associated with loss of muscle mass that may ultimately lead to sarcopenia, are summarized in figure 4.

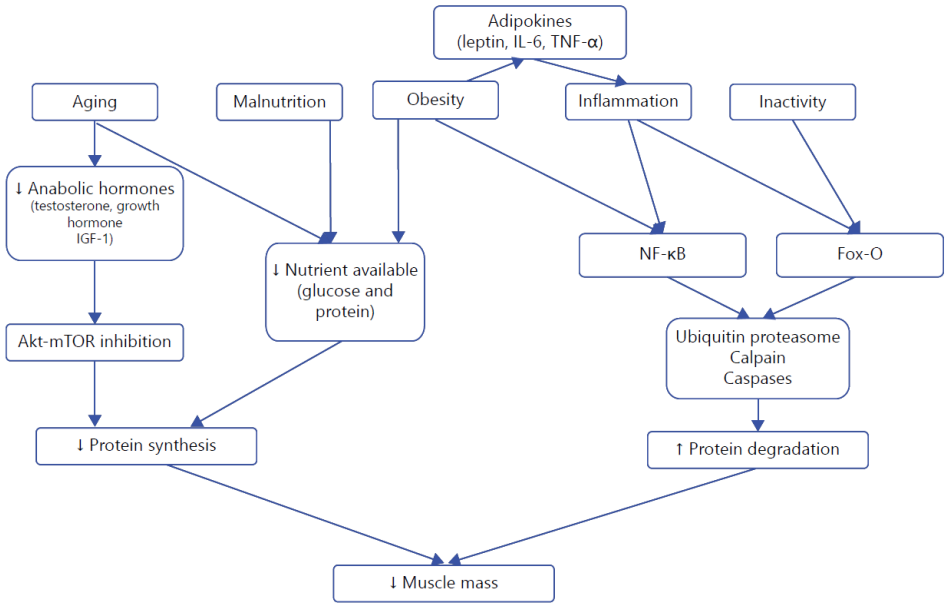


Figure 4. Factors associated with loss of muscle mass that may ultimately lead to sarcopenia. Reprint from Campins *et al*. [138], with permission from S. Karger AG.

1.4 Physical activity and health

Physically activity reduces the risk of developing chronic diseases such as CVDs, type 2 diabetes, some types of cancers and obesity [2, 139-142]. Exercise improves quality of life [143] and exercise capacity is a strong predictor of overall mortality rates regardless of health status and race [144]. The Norwegian Directorate of Health therefore recommends adults (18-64 yrs) to be moderately physically active for at least 150 min, or vigorously active for at least 75 min, throughout the week [145]. However, there seems to be a dose-response relationship between physical activity and health benefits in healthy, normally active subjects, increasing the health benefits with increased activity levels [139].

Physical exercise is divided into endurance training and strength training. Endurance training involves low-resistance exercises, such as walking, running and swimming, and strength training involves high-resistance exercises, such as weight lifting. Skeletal muscle, accounting for approximately 40% of the total body weight [146] is an extremely plastic tissue with a

remarkable ability to respond and adapt to environmental changes, such as exercise training [147] and nutritional modifications [148]. Long-term adaptations in skeletal muscle to endurance training are primarily apparent through increased mitochondrial biogenesis and enhanced aerobic metabolism [149], whereas long-term adaptations to strength training are accumulations of contractile proteins resulting in increased muscle mass (hypertrophy) and strength [150]. The molecular mechanisms responsible for adaptations to training are not fully elucidated, but may include changes in signaling (e.g. altered signaling of mTORC1), transcription (e.g. altered mRNA expression) and metabolic responses (e.g. altered body composition). Dietary manipulations to enhance adaptations to exercise have also been investigated [151]. It is well established that net protein synthesis is enhanced when exercise is combined with sufficient amounts of amino acids [152]. In addition, supplementation with BCAA has been shown to modulate the immune response after acute endurance training [153].

In young elite athletes, optimizing the recovery period to maximize the output of the following exercise session, which may only be hours away from the first exercise session, may be important. Whey protein supplements have been extensively used by athletes to promote a fast increase in muscle protein synthesis, as some research has shown that whey may be superior in promoting muscle protein synthesis compared to both casein and soy protein right after resistance exercise [154-157]. It is hypothesized that this effect is caused by the high leucine concentration in whey protein [129] and the fast absorption [155]. Whether the beneficial acute effects of whey protein on muscle protein synthesis are reflected in increased muscle mass, strength and performance are less clear.

Skeletal muscle is recognized as an important endocrine organ [158], and several hundred proteins are produced by skeletal muscle [159, 160]. Some of these are secreted in response to exercise, possibly exerting systemic effects [161]. In addition to increasing insulin sensitivity, reducing the level of triglycerides and blood pressure [162, 163], exercise has been shown to reduce the level of markers known to be involved in chronic low-grade inflammation [164-166]. However, the adaptations to exercise are highly dependent upon exercise intensity, type of exercise (endurance vs strength training), duration of the exercise session [167], training status, age and nutritional status [168, 169]. In addition, the acute effects differ from long-term adaptations to exercise [150].

1.4.1 Acute strength exercise – recovery of skeletal muscle

An acute exercise session represents a major challenge to whole body metabolism. During a high-load strength exercise session, there will be a sudden need for energy to the working skeletal muscle, and the breakdown of muscle glycogen stores to ATP and lactate is mainly provided by anaerobic metabolism [170]. Strength exercise also promotes protein synthesis [171], leading to muscle hypertrophy when regularly repeated [172]. Further, a whole range of molecules, often termed myokines, is produced within the skeletal muscle during exercise. Some of them will also enter the blood stream [159], thereby being able to influence other organ systems, among others the immune system [173-175]. Moreover, some of these molecules are known to be involved in the development of chronic low-grade inflammation, such as IL6 [67, 176-178]. IL6 is involved in the development of insulin resistance [179] and in the pathogenesis of atherosclerosis [180]. Simultaneously, and while induced after exercise, IL6 has been shown to promote insulin sensitivity and to increase fatty acid oxidation [181] as well as anti-inflammatory markers, such as IL10 and IL1RN [182-184]. Further, IL6 has been shown important for repair and regeneration processes in skeletal muscle after exercise [185, 186]. This apparent paradox may be explained by different roles of these molecules depending on whether they are temporarily released by working skeletal muscle during exercise, or constantly being released, e.g. by adipose tissue, in conditions such as chronic low-grade inflammation. Moreover, TNF, which is a central mediator of the inflammatory response, but less involved in acute response to exercise [182]. The acute response to an inflammatory response, such as sepsis, compared to an acute response to exercise is illustrated in figure 5.

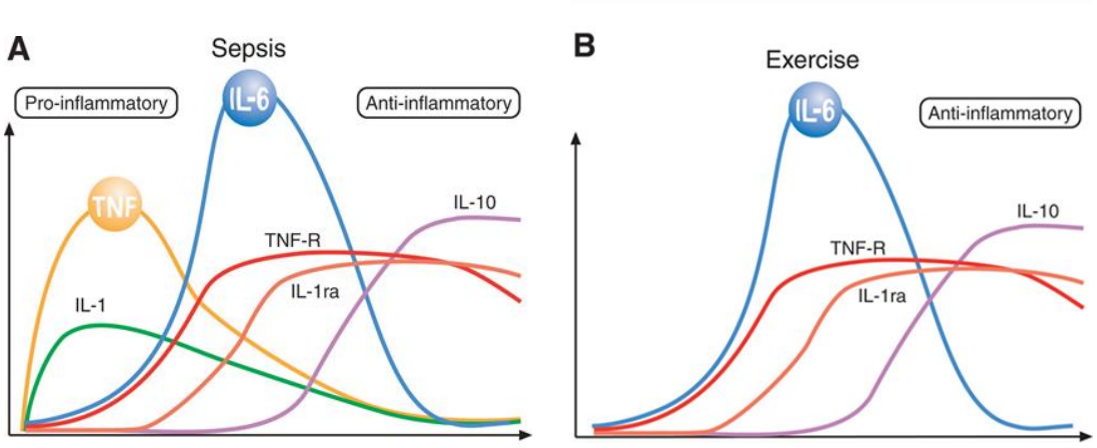


Figure 5. Acute responses to sepsis (A) and exercise (B) of markers involved in the immune system. Reprint with from Petersen and Pedersen [182] with permission from The American Physiological Society.

Aging has been shown to impair mTORC1 signaling [187] and recovery processes after acute exercise both in skeletal muscle [188-190] and the immune system [191], suggesting altered adaptations to exercise in older adults. However, there are also studies showing that older adults still have functional responses to exercise and that aging itself does not affect the response to exercise [192]. Further investigations are needed to understand the impact of aging on training adaptation.

1.4.2 Regular strength training – long term adaptations

The most visible adaptation to strength training is increased muscle mass (hypertrophy). Eight weeks of heavy strength training increased skeletal muscle mass with 3-4 % and muscle strength with 10-12 % in older adults [193], while one week in bed reduced muscle mass by approximately 2.5 % in healthy young men [194]. Thus, regular strength training is important to increase and maintain muscle mass in young as well as older adults to prevent sarcopenia [195-198]. Further, aging as well as diet have been shown to affect adaptations to training [169]. Combining protein supplementation with strength exercise has, for example, shown additional augmentation of muscle protein synthesis in older adults [199, 200]. The major underlying mechanism of muscle hypertrophy involve a positive net protein balance, mediated by changes in gene expression and protein levels of molecules involved in protein synthesis and breakdown

[201]. An illustration of how repeated bouts of exercise and regular training may affect mRNA expression and protein levels, is shown in figure 6 (modulated from [202]).

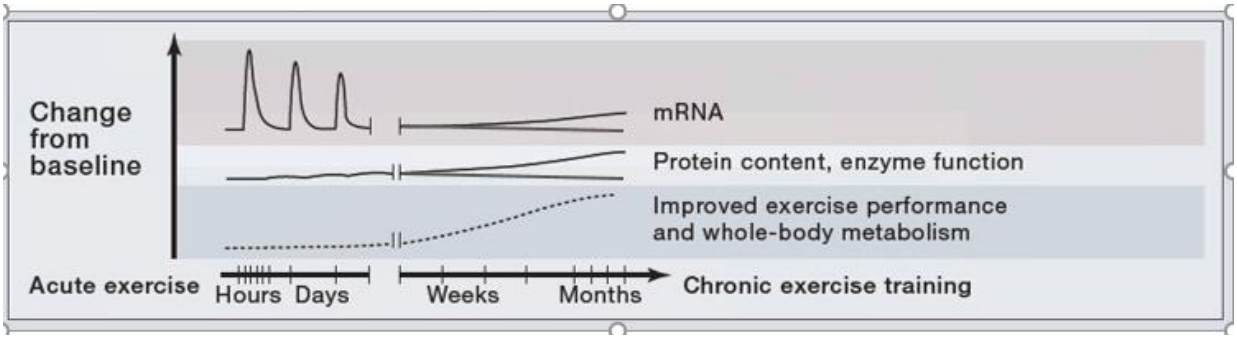


Figure 6. Illustration of changes in mRNA expression and protein content during acute, and after regular training. The mRNA expression or protein levels may increase or decrease during chronic training depending on mRNA transcripts investigated. Diet may be able to affect the mRNA expression levels, both acutely and chronically. Modulated from Egan *et al*, 2016 [202], with permission from Elsevier.

Strength training has also been shown to promote an anti-inflammatory milieu in the body [164, 203, 204], but the mechanisms underlying these effects are largely unknown. The reduction or redistribution of adipose tissue may be one explanation [164], another that the repeated exercise-induced spikes of IL6 increase the production of anti-inflammatory cytokines, such as IL10 and IL1RN, promoting an anti-inflammatory milieu in the body [164].

1.5 Gene expression studies in nutrition research

Variations in diet and other environmental factors, such as activity level, may only cause modest effects on measurable markers of health, and may therefore be difficult to observe [65]. Never the less, small acute changes may potentially be important in a life-long perspective, affecting homeostatic control and the risk of developing chronically related diseases [65]. Adaptations to environmental factors, such as dietary compounds or physical activity, involve an induction or reduction of several signaling pathways [6]. These signaling pathways will induce or inhibit gene expression levels at the transcriptional or translational level, or interfere with protein degradation. As illustrated in figure 7, environmental factors, such as diet and nutritional compounds, are able to interfere with gene expression levels either; a) directly, b) through transcription factors after modulated by metabolism or c) through stimulation of signaling

pathway(s) that ends with the induction of transcription factor(s), creating a ”signature” of the exposure [205]. These “signatures” can be studied in intervention studies to seek the molecular mechanism behind the exposure, to understand how these signals influence homeostasis and to look for early biomarkers [6, 206]. Advances in technology have expanded the possibilities to study the interactions of diet and health in a much more detailed and complex manner now than only a few decades ago [206].

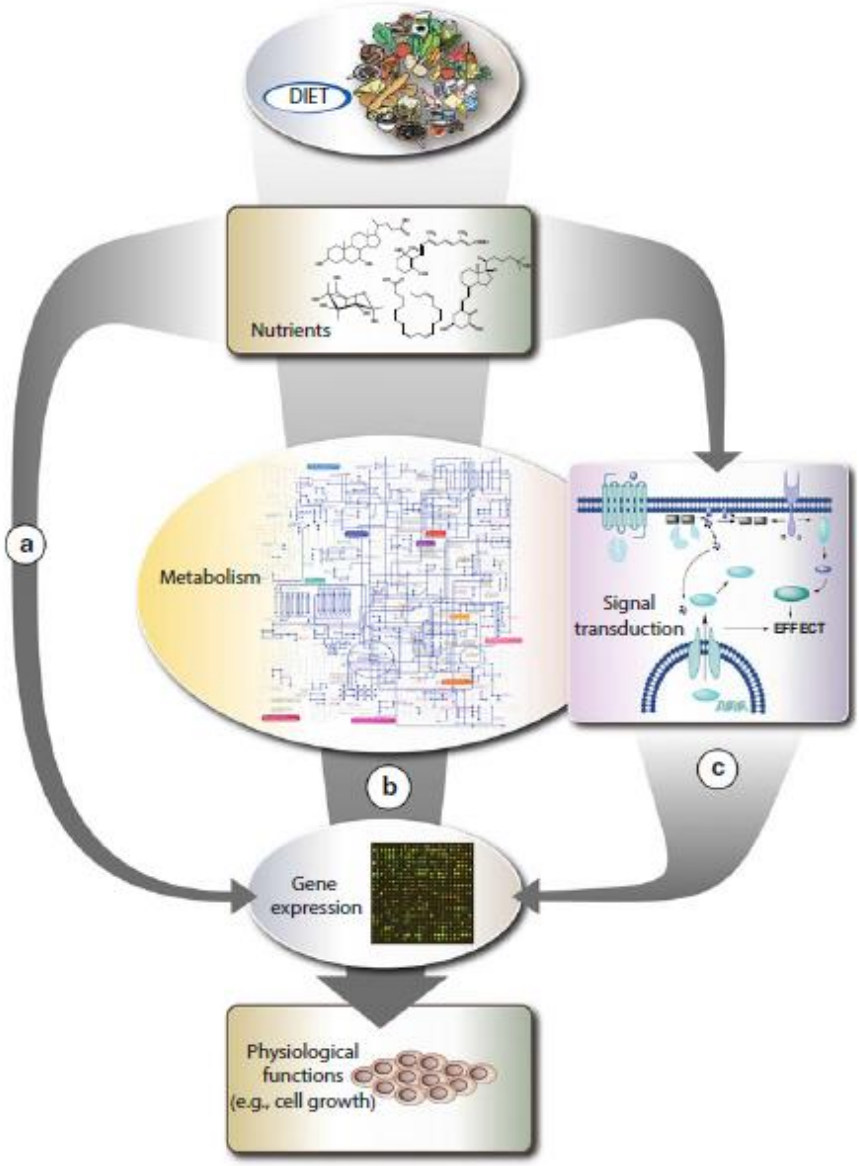


Figure 7. Diet may affect gene expression either directly (a) or indirectly via metabolism (b) or transcription factors (c), creating a signature of the exposure. Reprint from Carlberg *et al*, 2016 [205], with permission from Springer.

Human research has limited access to tissue, except for blood samples that are easily obtained. Blood samples are therefore frequently used in intervention studies. Similarly, PBMCs, which are circulating cells of the immune system and easily isolated from a blood sample, are more commonly used when studying changes in gene expression levels in intervention studies [207]. PBMCs, mainly consisting of monocytes and lymphocytes, have been shown to reflect hepatic regulation of cholesterol metabolism [208] and since PBMCs can migrate through the blood circulation and infiltrate various tissues, PBMC gene expression has been proposed to reflect metabolic and immune-related responses of adipocytes and hepatocytes [207]. Further, Liew and colleagues revealed that over 80% of the genes expressed in PBMCs were co-expressed in other tissues, such as liver, adipose tissue and skeletal muscle [209]. Changes in PBMC gene expression have therefore been suggested as a good model to study responses to nutritional interventions in several tissues [209, 210]. Gene expression analysis may therefore be important tools in intervention studies to detect early signs of homeostatic dysregulation not yet manifested into an altered phenotype [206].

2 Aims

The overall aim of this project was to investigate health effects of dairy protein on muscle mass, muscle strength and markers known to be involved in chronic low-grade inflammation in older adults (≥ 70 yrs).

Specific aims were to investigate if:

- intake of 20 g milk protein served together with breakfast and evening meal could improve muscle mass, muscle strength and functional performance in older adults (paper I)
- intake of 20 g milk protein served together with breakfast and evening meal could improve markers of chronic-low grade inflammation among older adults (paper II)
- intake of 20 g milk protein, WPC80 or native whey after an acute session of high-load strength exercise could differently alter the acute response in skeletal muscle and PBMCs, and to compare this response in young and older adults (paper III)
- intake of 20 g milk or native whey protein twice a day, combined with eleven weeks of high-load strength exercise, could differently alter mRNA transcripts of immune-related genes in skeletal muscle and PBMCs in older adults (paper IV)

3 Subjects and methods

This work is based on two double-blind randomized controlled trials and one double-blind (partial) crossover study conducted at the HiOA and the NIH from the fall 2013 through the spring 2015. Table 2 provides an overview of the study populations and study designs.

Table 2. Overview of study populations and study designs.

Design	Population	Intervention	Duration	Analysis	Paper
Double-blind randomized controlled trial (study 1)	Older (≥70 yrs), men and women (n=36)	Protein-enriched milk (2 x 0.4 L/d, 20 g protein) or isocaloric carbohydrate drink (2 x 0.4 L/d) with breakfast and evening meal.	12 weeks	DXA, muscle strength, functional tests	I
				PBMC gene expression, serum IL6, TNF, sTNFRSF1A	II
Double-blind (partial) crossover study (study 2)	Young (20-40 yrs) and older (≥70 yrs) men and women (n, young=24, n, older=17)	Milk protein, WPC80 or native whey protein (2 x 20 g protein/d) ingested after a standardized bout of strength exercise.	1 day (milk) 1 day x 2 (whey group)	Skeletal muscle and PBMC gene expression, serum IL6	III
Double-blind randomized controlled trial (study 3)	Older (≥70 yrs), men and women (n=24)	Milk protein or native whey (2 x 20 g protein/d) ingested in the morning and evening in combination with strength training 3 times per week. Standardized strength exercise session before and after the intervention.	11 weeks	Skeletal muscle and PBMC gene expression, serum IL6	III+IV

3.1 Subjects

Subjects were recruited through posters, newspapers, Facebook and exhibition stands during the fall of 2012 and throughout the fall of 2014 by staff at the University of Oslo (UiO), HiOA and NIH. Inclusion and exclusion criteria are listed in Table 3.

Table 3. Inclusion and exclusion criteria in the studies performed.

Double-blind randomized controlled trial Paper 1		**Double-blind (partial) crossover study** Paper II		**Double-blind randomized controlled trial** Paper III+IV	
Inclusion criteria	*Exclusion criteria*	*Inclusion criteria*	*Exclusion criteria*	*Inclusion criteria*	*Exclusion criteria*
Age ≥ 70 yrs	Allergic or intolerant to milk	Age 20-40 and age ≥ 70 yrs	Allergic or intolerant to milk	Age ≥ 70 yrs	Allergic or intolerant to milk
Men and women living at home	Unable to perform the physical tests	Healthy men and women living at home	Allergic to local anestesia	Healthy men and women living at home	Allergic to local anestesia
Reduced grip strength, gait speed, timed step stair test, timed five times sit to stand test	MMSE score < 24	Young: strength training at least once a week the last six months Older; recreationally active	Not able to read and speak Norwegian	Unfamiliar with strength exercise	Not able to read and speak Norwegian
Willingness to keep physical activity levels stable	MNA < 17, weight change		Heart attack the last 6 months		Heart attack the last 6 months
Stable body weight last three months	Intake of milk and/or yoghurt ≥ 0.4 L/day		BMD under 0.84g/cm^2 in L2-L4		BMD under 0.84g/cm^2 in L2-L4
	Alcohol consumption ≥ 40 g alcohol/d		Used glucocorticosteroids for the last 6 months		Used glucocorticosteroids for the last 6 months
	Subjects with diabetes type I or II or HbA1c ≥ 6,5%		Uncontrolled hypertension		Uncontrolled hypertension

Table 3. Inclusion and exclusion criteria in the studies continued.

Double-blind randomized controlled trial Paper 1		**Double-blind (partial) crossover study** Paper II		**Double-blind randomized controlled trial** Paper III+IV	
	Exclusion criteria		*Exclusion criteria*		*Exclusion criteria*
	Severe inflammation, COPD High blood pressure (> 180/105 mmHg) CVD the last six months Cancer last 3 yrs eGFR < 45 ml/min CRP level ≥ 10 mg/L, and > three times the reference limit of ALAT and/or AST Unwillingness to stop using Ca-supplements		Used dietary supplements Unable to carrying out the training program		Used dietary supplements Unable to carrying out the training program

Abbreviations used in table; ALAT, alanine aminotransferase; AST, aspartate aminotransferase; BMD, bone mineral density; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; L2, vertebrae lumbar 2; MMSE, mini mental state examination; MNA, mini nutritional assessment;

3.2 Test products

All study products were produced and provided by TINE SA (Norway). In study 1, commercially available protein-enriched milk was used, whereas the control drink was produced for the purpose of the intervention study. The protein-enriched milk provided on average 5.1 g protein, 4.9 g carbohydrate, < 0.1 g fat and approximately 174 kJ (41 kcal)/100 g. Each serving (0.4 L) contained 20 g protein. The control drink was isocaloric consisting of carbohydrate (maltodextrin, sugar and xantan gum) only. E171 was added to the control drink to give it a milky appearance.

In study 2, three different liquid products were made for the purpose of the study. The different milk proteins were incorporated into a drink together with cream milk, sugar, aroma and water. The products were isocaloric providing on average 3.2 g protein, 6.2 g carbohydrate, 1.1 g fat and approximately 202 kJ (48 kcal)/100 g, containing 20 g protein per 638 ml. The only difference between the products tested was the protein source, which was regular milk protein, WPC80 or native whey protein (Table 4). All ingredients used were commercially available, except for native whey that was especially produced for the purpose of this study. To mask the taste of the different products, raspberry flavor was added to all products.

In study 3, two different powders were made for the purpose of the study; one was based on regular milk protein and the other of native whey powder bought from Lactalis Industry (France). The different milk proteins were incorporated into a powder together with cream milk, sugar and aroma. The powders were to be dissolved with approximately 0.5L water before use. The nutrient composition was identical to the products used in study 2, but a commercially available powder of native whey was used instead of the liquid produced native whey used in study 2. To mask the taste of the different test products, vanilla flavor was added to both powders.

We chose to make test products containing 20 g protein per serving in all three studies as literature indicated that this amount was sufficient to maximally stimulate muscle protein synthesis [211, 212]. In addition, participants should be able to ingest the volume provided to ensure the validity of the results.

Similar packaging was used to ensure blinding of both participants and research staff. Labeling with color codes or ID numbers was used to ensure that participants received the same products throughout the studies.

The amino acid profile of protein-enriched milk, regular milk, WPC80 and native whey protein used in the different studies are shown in Table 4. Values are presented as mean values based on analysis from at least two batches performed at an accredited laboratory (Eurofins Food & Feed Testing, Moss, Norway).

Table 4. Amino acid composition of test products.

Amino acids (% of tot aa)	**Protein-enriched milk (study 1)**	**Low-fat milk (study 2+3)**	**WPC80 (study 2)**	**Native whey (study 2)**	**Native whey (study 3)**
Alanine	3.1	3.2	4.8	4.8	4.4
Arginine	3.3	3.3	2.4	2.5	2.8
Aspartate	7.5	7.5	10.6	11.3	10.3
Cysteine	0.7	0.8	2.1	2.6	2.3
Phenylalanine	4.7	4.7	3.3	3.7	3.9
Glutamic acid	20.6	20.4	17.1	16.9	17.8
Glycine	1.8	1.9	1.9	1.9	1.8
Histidine	2.6	2.7	1.9	2.0	2.3
Isoleucine	4.8	4.9	6.0	5.4	5.2
Leucine	9.4	9.6	10.3	12.2	11.3
Lysine	8.1	8.2	9.2	10.2	9.5
Methionine	2.5	2.5	2.1	2.1	2.4
Proline	9.2	9.7	6.4	5.0	5.9
Serine	5.5	5.6	5.4	4.6	4.8
Threonine	4.3	4.4	7.0	5.0	4.8
Tyrosine	4.5	3.5	2.1	2.5	3.3
Valine	6.0	6.0	5.7	5.1	5.5
Tryptophan	1.4	1.3	1.7	2.1	1.9
EAA	43.9	41.4	45.3	45.9	44.5
BCAA	20.2	20.5	22.0	22.7	22.0

3.3 Ethics

All studies conducted were approved by the Regional Committees for Medical and Health Research Ethics, Health Region South East, Norway, and performed according to the Declaration of Helsinki (last amended 2008). All participants received detailed written and oral information about the projects before deciding upon participation and were eligible to withdraw from the study at any time. Written informed consent was obtained from all participants.

4 Summary of papers

4.1 Paper I

Intake of a protein-enriched milk and effects on muscle mass and strength. A 12-week randomized placebo controlled trial among community-dwelling older adults

In the first paper, we aimed to investigate the effects of providing 0.4L protein-enriched milk (20 g protein) with breakfast and evening meal on muscle mass, muscle strength and functional performance in older adults (≥ 70 yrs) with reduced strength and/or performance.

We found that chest press was significantly improved in both groups, but no significant differences were observed between the two groups. Further, no significant differences were observed between groups for leg press or muscle mass, nor in the functional performance tests. Serum total- and low-density lipoprotein cholesterol were significantly decreased in the protein group after 12 weeks, with no significant change between the two groups. No adverse effects on kidney function were observed in the protein group.

In summary, we were not able to show effects of an increased protein intake during breakfast or evening meal on muscle mass, muscle strength or functional performance in older adults with reduced strength and/or performance compared to an isocaloric intake of carbohydrate.

4.2 Paper II

Consumption of protein-enriched milk has minor effects on inflammation in older adults – a 12-week double-blind randomized controlled trial

Using the same study population as in paper I, we aimed at investigating whether intake of protein-enriched milk (20 g protein/0.4L) twice a day for 12 weeks could influence markers of inflammation. We measured serum levels and mRNA expression levels in PBMCs of selected inflammatory markers at baseline and after the intervention period.

After the intervention period, we observed significant differences in mRNA expression levels of *nuclear receptor subfamily, group H, member 3 (NR1H3)* and *interferon gamma (INFG)* between the two groups. The expression of *NR1H3* and *INFG* increased slightly in the milk group, while a small decrease was observed in the control group. Further, the mRNA expression level of *tumor necrosis factor receptor superfamily member 1A (TNFRSF1A)* was significantly reduced, whereas the mRNA expression level of *dipeptidyl-peptidase 4 (DPP4)* was significantly increased in the control group, but with no differences between the groups. The serum level of TNF increased significantly in the control group, whereas the serum level of TNFRSF1A increased significantly in both groups, with no significant differences between the two groups.

In conclusion, consuming protein-enriched milk for 12 weeks had minor effects on inflammatory markers in older adults compared to an isocaloric carbohydrate drink.

4.3 Paper III

Gene expression is differently regulated in skeletal muscle and circulating immune cells in response to acute high-load strength exercise

The aims of this paper were to investigate the effects of regular milk protein, WPC80 and native whey on the acute response to high-load strength exercise and to compare this response in skeletal muscle and PBMCs of young (20-40 yrs) and older adults (≥ 70 yrs).

We found that an acute bout of high-load strength exercise altered many of the genes measured, but there were no significant differences in the response between subjects who ingested WPC80 compared to native whey. Nor were there any significant differences in mRNA expression levels between the milk and whey group, when combining the two whey groups. When comparing mRNA expression levels in skeletal muscle and PBMCs, we observed three different expression patterns; i) mRNA transcripts increased significantly in skeletal muscle only ii) mRNA transcripts increased significantly in both skeletal muscle and PBMCs, but the increase was greater in skeletal muscle than in PBMCs and iii) mRNA transcripts were similarly expressed in skeletal muscle and PBMCs. These expression patterns were observed for both young and older adults, but the mRNA response of *IL8*, *CCL3*, *IL1β* and *IL10* were significantly attenuated in PBMCs of older adults compared to the response in younger adults.

Altogether, the results showed that an acute bout of high-load strength exercise induced both overlapping and unique responses in mRNA transcripts in skeletal muscle and PBMCs, indicating tissue specific functions of skeletal muscle and PBMCs in response to acute strength exercise. There were no differences in the response depending on the drinks provided. However, attenuated responses in some mRNA transcripts in PBMCs were observed in older compared to younger subjects after exercise, suggesting an altered adaptation to exercise in older adults.

4.4 Paper IV

Eleven weeks of strength training decreased the expression of immune-related genes in older subjects independent of protein supplement type; a randomized controlled trial

In this study, we investigated if supplementation with milk or whey protein in combination with high-load strength training could differently alter mRNA expression levels of immune-related markers in skeletal muscle and PBMCs of adults >70 yrs.

We found significantly reduced mRNA expression levels of *IL6, IL8, CCL3* and *NR1H3* in PBMCs after the intervention period, whereas the mRNA expression of *TLR2* increased. In skeletal muscle, the mRNA expression of *PPARGC1A* and *PPARGC1B* decreased significantly, whereas the mRNA expression of *CCL2, CCL5, TLR2, TLR4* and *HIF1A* significantly increased after the intervention. The decreased levels of *IL6, IL8* and *CCL3* in PBMCs may promote an anti-inflammatory milieu in the body, whereas increased levels of immune-related mRNA transcripts in skeletal muscle may be related to resolution and adaptation processed related to the combined training and supplementation intervention. We found no significant differences in circulating CRP and IL6 after the intervention period. Furthermore, the consumption of whey and milk proteins had similar effects on mRNA expression levels after strength training in skeletal muscle as well as in PBMCs.

In summary, combining strength training and protein supplementation reduced the mRNA expression levels of several immune-related mRNA transcripts in PBMCs, whereas in skeletal muscle, we found an increased level of immune-related mRNA transcripts. The impact of these changes is unclear and needs further investigations. Furthermore, we observed no differences in mRNA expression levels depending on supplements provided. Thus, we concluded that native whey and regular milk protein exerted similar effects on the mRNA transcripts investigated, both in PBMCs and in skeletal muscle.

5 Discussion

5.1 Methodological consideration

5.1.1 Subjects

The majority of subjects included in these studies were recruited from the local community. Many of the young subjects recruited to the double-blind (partial) crossover study (the acute exercise study) were enrolled at NIH, and several of the older adults were recruited from a nearby activity center. Compared to the general population, these subjects may be more likely to have a special interest in sports, possibly also diet, potentially being more physically active and eating healthier than the general population. In the training study, subjects were untrained prior to inclusion. Recruitment was especially challenging among older adults with reduced physical performance. All subjects were living at home, but had to travel to the test facilities by themselves, which may have provided us with the healthiest subjects within the target group.

The local recruitment, the recruitment of active and potentially healthier subjects and the relatively low number of subjects included in the studies should be considered when interpreting the results and in relation to the generalization of the results to other parts of the population.

5.1.2 Study design

We conducted two human randomized controlled trials and one randomized (partial) crossover study, both double-blind, as part of a larger project where the purpose was to document the health effects of a new high-protein ingredient (native whey) on muscle mass, muscle strength and inflammation. Two studies included physical exercise, whereas one study contained no exercise. Double- blind randomized controlled trials were chosen as study design as they are ideal when performing human studies trying to establish a cause and relationship between an intervention and the outcome [213, 214]. Further, dietary registrations were performed and adherence to the study protocol closely followed in all three studies to ensure validity of the results [214]. To counteract substitutions of nutrient rich foods due to the high volume of the test drinks (2 x 0.4L), participants were advised to remove other drinks from their diet if necessary, not foods. To try making the drinks easier to consume, we provided all participants

with suggestions for alternative ways to use the test drinks at inclusion. There were still participants that found the volume difficult to ingest and some that did not like the test drinks, but we have no reason to believe that this influenced the outcome of the study (e.g. skewed withdrawal from the study). Ideally, a series of equally high-protein products with different tastes should have been provided, but this was not possible due to extra production costs and from a logistically point of view.

In the studies performed at NIH, all three groups performed the exercise and all three groups received milk protein. With this design, we were able to compare possible differences between the three test products, but we were not able to obtain any information about possible effects of milk protein *per se* combined with exercise, or to distinguish the effects of exercise alone. To adequately address these questions, a fourth group, where participants did not receive protein, only exercised, should have been added to the study. Further, combining the results from such a study with results obtained from the study at HiOA could have provided us with valuable information about the singular effects of protein with or without exercise (if native whey had been used in that study performed at HiOA).

Timing of sampling in the acute exercise study at NIH was decided based on the primary aim of the studies and may not have been optimal for the analysis of inflammatory markers in the tissues chosen [215]. Some time is needed before changes can be observed in target tissues or cells, as products need to be digested, absorbed and distributed to the relevant tissues and cells before changes can be detected. The first time point might therefore have been too early to observe any changes in mRNA expression levels caused by the added protein.

5.1.3 Test products

All test products were produced by TINE SA (Oslo, Norway). Due to technological challenges, TINE was unable to produce enough native whey to cover all three studies. We therefore decided to use commercially available protein-enriched milk in the study performed at HiOA and compare it to an isocaloric carbohydrate drink (study 1). By this change we were still able to investigate possible effects of a milk-based drink with a high content of protein, but we lost the opportunity to compare the effects between subjects who exercised (included at NIH) with those who did no exercise (included at HiOA). In study 2 (acute exercise study performed at NIH), we used native whey protein from TINE, while a commercially available powder from

Lactalis Ingredients (France) was used in the training study (study 3). The two native whey ingredients had a similar amino acids composition (Table 4).

In all studies, we found it important to make isocaloric control drinks to exclude possible effects of differences in energy content. We also flavored the products to mask the taste. Further, all test products made for the studies at NIH contained the same amount of macronutrients, including protein, leaving the protein source the most variable factor between whey and regular milk protein. Products contained 20 g protein per serving as 20 g high-quality protein has been shown to be the optimal dose for stimulation of muscle protein synthesis after strength exercise [216]. However, later evidence has suggested that an optimal dose for older adults may be higher than 20 g protein [217].

Native whey contains some more leucine that WPC80 and further differed from WPC80 by the production method. While WPC80 is a by-product from cheese production, native whey was produced by a two-step cold membrane process, directly from unpasteurized milk. To ensure the microbiological quality of the native whey protein, we used filtration methods to remove microorganisms if present. The native whey powder from Lactalis Ingredients was similarly produced as the liquid native whey, but dried to form a powder.

Participants in the acute exercise study experienced taste and volume (0.63L) of the test drink differently. Most people consumed the products within the time limit (5 min), but there was a general consensus among participants that the test products did not taste very well. The flavor of the test products was therefore successfully changed to the training study.

5.1.4 Timing of supplements

In many countries, including Norway, dinner provides the highest amount of protein during the day [218] as illustrated in figure 8. No recommendation for the distribution of protein is made [26], but it is hypothesized that each meal should contain 25-35 g protein to maximally stimulate protein synthesis [219]. Similarly, a randomized 7-d crossover feeding study, where healthy young men and women were recruited, showed that consuming a moderate amount of protein at each meal stimulated 24-h muscle protein synthesis more effectively than a skewed intake of protein [220]. In study 1, we therefore encouraged participants to ingest the test drinks with breakfast and the evening meal to reach a protein intake above 20 g per meal, and to have ≤ 11 hours between the evening and the morning test drink to reduce night fasting.

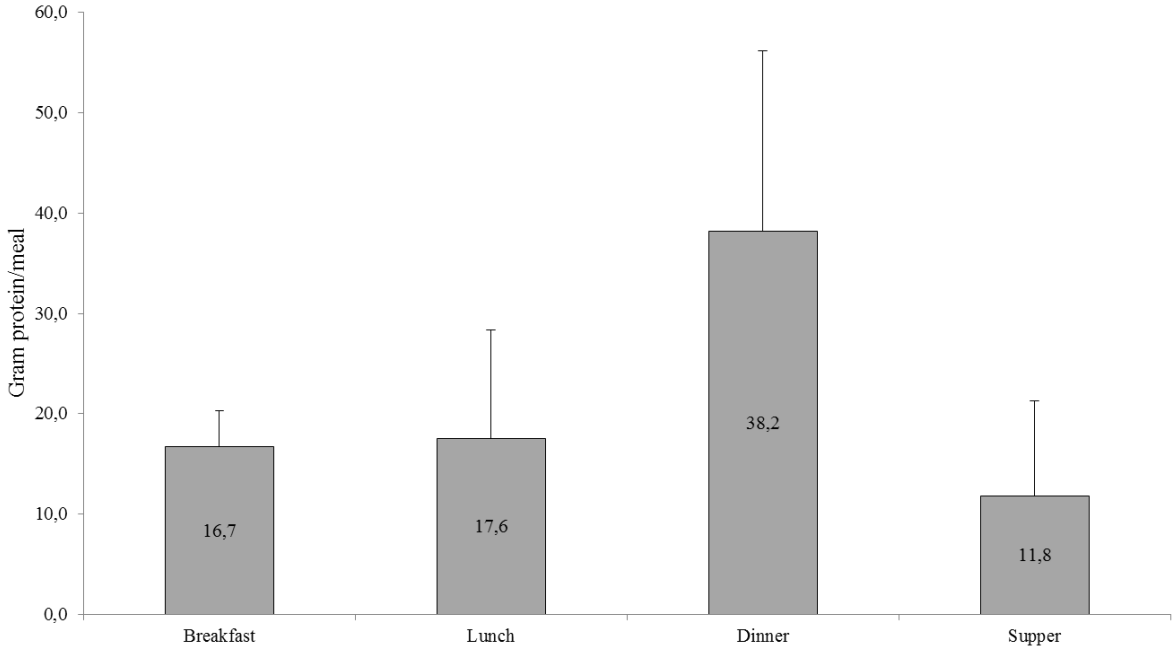


Figure 8. Average protein intakes to each meal during a 24-h period in 420 adults > 70 yrs in Skedsmo municipal, Norway (Ottestad et al, unpublished data). The protein intake exceeds 20 g to dinner only.

The importance of distributing the intake of protein throughout the day for maximal stimulation of muscle mass and strength has lately been questioned [24, 136]. In the present study (study 1), we were not able to show that an evenly distribution of protein through the day were important to increase muscle mass and strength (paper 1).

5.1.5 RNA extraction from skeletal muscle

We used a newly established method at our department for RNA extraction from skeletal muscle. The method was thoroughly tested on skeletal muscle from mice to make sure that the method worked properly. The isolation of RNA from the acute exercise study went without extensive problems, but we experienced extensive methodological challenges with RNA extraction in the training study. We adjusted the protocol slightly when extracting RNA in the training study to be able to place samples on the QiaCube. These changes were successfully tested using skeletal muscle biopsies from mice prior to applying on the human samples. These methodological challenges resulted in a substantial loss of samples in the training study, but we are confident that data from the samples included are valid as the quality, measured with

Nanodrop-1000 (NanoDrop Technologies, Inc., Delaware, USA), and the quantity, measured with Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., California, USA) of the samples were satisfactory (RIN-value above 6 for all skeletal muscle samples). In addition, the loss of samples was random and equal between groups.

5.1.6 Selection of genes

We used 48 wells Taqman Low Density Array (TLDA) cards for the mRNA expression analysis, and made a selection of mRNA transcripts to analyze. The selection was made based on suggested associations between milk, milk products and inflammatory markers [221, 222], mRNA transcripts associated with inflammatory markers in PBMCs [223] and regeneration and adaptation in skeletal muscle [224]. We mainly focused on mRNA transcripts associated with NF-κB-signaling and metabolic diseases [177, 225], including genes linking inflammation and metabolism [226]. In study 1 (paper II), we also included mRNA transcripts known to be involved in bone metabolism [227, 228], myofiber denervation [229], energy metabolism [230, 231] and protein synthesis and breakdown [232, 233].

5.1.7 PBMC as a surrogate model

PBMCs are exposed to both endogenous and exogenous stimuli and are continuously interacting with other cells and organs within the body. Subtle changes occurring in these cells or organs may trigger specific changes in PBMC gene expression reflecting the initiating stimulus [209, 234]. PBMCs as a model system has mostly been used when studying the impact of dietary components on inflammation in relation to the development of CVDs [207, 235], but has also been used in cancer diagnostics and to determine the response to toxin exposure [236]. In paper III and IV, we showed that some mRNA transcripts were regulated similarly in skeletal muscle and PBMCs, whereas other mRNA transcripts showed a more unique pattern, suggesting tissue specific functions in response to acute exercise combined with protein supplementation. This was supported in a study were adipose tissue and PBMC gene expression were found to complement, rather than directly reflect each other [234]. Thus, is seems important to evaluate the physiological processes in each tissue/cells in specific physiological situations, such as exercise, to be able to determine if PBMCs can be used as a reliable surrogate model or serve as a complement to the findings in other tissues/cells.

So far, PBMC gene expression analysis have primarily been used when investigating possible effects of fats, fruits, vegetables and herbs on markers of chronic low-grade inflammation [209]. How dietary protein affects gene expression in PBMCs has not been extensively investigated, and needs further investigations.

5.1.8 Skeletal muscle as a source of muscle fibers

In the present studies we collected muscle biopsies from *m. vastus lateralis*. In addition to muscle fibers, muscle biopsies from *m. vastus lateralis* will contain other cells types, including immune cells, endothelial cells, fibroblasts and nerve cells [77]. We are therefore not able to distinguish which cell type that contributed to the altered mRNA expression levels observed in skeletal muscle in the present studies. The results from our analysis will provide information about the united response from several cells types within the skeletal muscle, with the skeletal muscle being the dominant cell type.

5.1.9 Statistical considerations

Power calculations were made for the primary outcomes of the studies. We were unable to recruit this number of participants, but the number of participants included equals the number included in similar studies exploring changes in mRNA expression levels of inflammatory related genes in response to exercise or diet [237-240]. The mRNA expression data were not normally distributed. We tried transforming the data by $\log_{10}$ and $\log_2$ transformation, but none of these transformations made the data normally distributed. We therefore decided to use non-parametric tests. Due to the explorative approach of these studies, we decided not to correct for multiple testing, being aware of the increased risk of detecting false positive results (type 1 error). The results obtained were therefore interpreted with caution, especially when only observing few statistically significant results.

5.2 Discussion of main results

5.2.1 Increased protein intake in older adults – effects on muscle mass, muscle strength and inflammation

We were not able to show that additional intake of dairy protein, ingested with the morning and evening meal (2x20 g/d), increased muscle mass, muscle strength, functional performance (paper I) or substantially altered levels of inflammatory markers (paper II) in older adults after 12 weeks of protein supplementation compared to an isocaloric carbohydrate drink. The non-significant differences in muscle mass and strength observed are both supported [137, 241, 242] and opposed by others [243, 244]. It has been suggested that muscle protein synthesis is stimulated by EAAs in a dose dependent matter [245], reaching a plateau at 15 g EAAs per meal [246] or approximately 20 g high quality protein [22]. Further, leucine has been shown the most potent amino acid in enhancing muscle protein synthesis [129]. Previous analysis have shown that 6.7 g EAA enriched with 26 % leucine did not increase muscle protein synthesis in older adults after a meal, whereas the same amount of EAA with 41 % leucine did [247]. Despite a high intake of protein in the present study (1.4 g protein/kg body weight vs 0.9 g protein/kg body weight), the amount of EAAs provided through consuming the test drink was not more than ~ 9.5 g. Moreover, analysis of the dietary registrations showed that participants had an adequate dietary intake of protein before entering the trial (1.0 g protein/kg body weight), possibly preventing further increase in muscle protein synthesis [137, 242]. To maximally stimulate muscle mass in older adults, it has been suggested that each meal should contain 25-30 g protein (2.5-2.8 g leucine) [219] and be evenly distributed through the day [220]. However, similar anabolic benefits from serving one meal high in protein per day have been observed when measuring the whole body anabolic response, not only muscle protein synthesis [24, 136, 248], indicating that an evenly distribution of proteins through the day may be unnecessary. Further, no plateau effect has been observed when estimating net anabolic response, including both muscle protein synthesis and muscle protein breakdown [24]. Even though no changes in muscle mass or strength were observed between groups, we observed improvements in chest press when combining the groups, indicating that increased energy intake per se may have been beneficial. This may be explained by the protein-conserving influence of dietary energy that potentially inhibits muscle protein degradation, but also the stimulation of protein synthesis [249]. Another explanation for the observed increase in chest press may be related to the test-retest phenomenon, as participants were not made familiar with the strength tests prior to the

first test day. The conflicting results from studies in this field may partly be explained by variations in study designs, composition of the protein supplements, adherence to the interventions and baseline health and nutritional status [214].

Elevated levels of circulating inflammatory markers, such as IL6 and TNF, are often observed in older adults [82], and are associated with loss of muscle mass and strength [91, 250]. Further, intakes of dairy products have been observed inversely associated with levels of inflammatory markers [96, 251]. It was therefore interesting to investigate if the intake of protein-enriched milk could lower markers of inflammation in older adults. Overall, the intake of protein-enriched milk did not alter levels of inflammatory markers compared to the isocaloric control drink, but we did observe a statistically different expression of two genes, *NR1H3* and *INFG*. An upregulation of *NR1H3* is linked to increased reverse cholesterol transport [252] and reduced inflammation [253], whereas an upregulation of *INFG* may induce a Th-1 cytokine release, increasing inflammation [254]. The physiological explanation of these differences is unclear, but leucine may be a precursor for lipid biosynthesis in skeletal muscle [255], possibly contributing to the difference in *NR1H3* gene expression levels between the two groups. Further, we observed increased mRNA expression of *PDK4* in the group receiving carbohydrates, which was consistent with the increased intake of carbohydrates. We observed that the mRNA expression level of *TNFRS1A* decreased in PBMCs in the carbohydrate group, whereas the serum concentration of TNFRS1A increased, illustrating the complexity of interpreting the human metabolism. Initially, these results seem contradictory. However, each tissue and cells have different physiological functions in a given situation and at a given time point, possibly explaining part of the opposing results. In addition, mRNA expression analyzes reflect the transcription in that specific tissue only, while circulating levels reflect the impact of several tissues and cells within the body. Further, altered mRNA expression levels will not always correspond to protein abundance because protein levels are determined by regulatory input from synthesis to degradation [206].

No safe upper limits for dietary protein intake have been established for healthy adults, but no adverse effects have been observed after long-term intake of 2 g protein/kg body weight/day [9, 26]. Diets high in protein do not seems to present any risk as long as the protein comes from foods, not amino acid supplements [8, 20], which results from the present study also indicate.

The combined effects of protein supplementation and strength training on muscle mass and strength are also investigated in this project (the primary aim of study 2 and 3), but are outside

the scope of the present thesis. However, based on already published literature, the optimal way to increase muscle mass and muscle strength to promote healthy aging seems to be through physical activity, mainly strength training, combined with ensuring proper energy balance, an optimal intake of protein [22, 46] and adherence to a healthy diet [46, 256]. However, further research is needed to identify the optimal training regime and protein intake for different age groups, and during different life-time situations.

5.2.2 Effects of protein and exercise on gene expression levels

Not only quantity, but also quality, are important for an optimal response to protein intake in young as well as in older adults [56]. Whey protein is absorbed fast [45] and has been shown superior to casein in stimulating muscle protein synthesis following an acute bout of resistance exercise in both young (22.8±3.9 yrs) [154] and older men (74±6.1 yrs) [157]. Casein aggregates in the stomach and shows a slower digestion and absorption rate than whey protein [45]. Based on the absorption rate of whey and casein, whey protein has been suggested to be more beneficial than casein to limit protein losses during aging [257]. The proposed effect may be caused by the fast increase of leucine in the blood stream when ingesting whey protein, compared to a more steady release of leucine to the blood stream when ingesting casein [129]. However, acute effects may differ from long-term effects as illustrated by Candow and colleagues who found minimal beneficial effects in lean tissue mass and strength after six weeks of supplementation with either whey or soy protein in combination with resistance training [258].

Different absorption rates, and possibly the different compositions of whey protein and regular milk protein may potentially also affect immune cells. However, we were not able to show that different source of milk protein differently altered the mRNA response neither in skeletal muscle nor in PBMCs (paper III), except from the expression of *CXCL16* in PBMCs in the training study (paper IV). CXCLs are able to control migration and residence of all immune cells, and CXCL16 is proposed to have dual functions in inflammation and homeostasis [259]. However, further analysis is needed to evaluate the validity of differences in *CXCL16* mRNA expression (as no correction for multiple testing was performed) and to explore the physiological relevance of this change. The present results indicate that milk and whey proteins exert similar effects on adaptation to acute strength exercise as well as strength training.

Independent of the protein supplements provided, the acute strength exercise session induced a whole range of metabolic responses, both in skeletal muscle and PBMCs in young as well as older adults (paper III). Both similar and unique responses were observed after the acute exercise session, suggesting that PBMCs may reflect the response in skeletal muscle for some mRNA transcripts, but not for others. Skeletal muscle and PBMCs are likely to exert different functions during exercise, which may cause different mRNA expression profiles in skeletal muscle and PBMCs. The temporarily increase of IL6 in skeletal muscle and plasma after exercise has been shown to promote insulin sensitivity, glucose uptake and fatty acid oxidation [260-262], affect satellite cells and promote myogenic lineage progression [185, 186], whereas TNF and IL1β may play a role in promoting myoblast proliferation [263], and inhibiting myoblast differentiation [264]. These processes may be important contributors to skeletal muscle adaptations. We observed no changes of *IL6*, *TNF* and *IL1β* mRNA expression in PBMCs after acute exercise, indicating a less important role of these genes in PBMCs after acute exercise compared to skeletal muscle. However, we observed a significant increase in mRNA expression levels of *IL10* and *IL1RN* after exercise in PBMCs as well as skeletal muscle. The increased mRNA expression levels of these genes may be a result of the increased level of IL6 observed in skeletal muscle after acute exercise [77, 182-184, 265]. IL6 in skeletal muscle may, however, also be increased by the activation of the c-Jun N-terminal kinases (JNK) pathway, possibly independent of the NF-κB-pathway [266] as illustrated in figure 9. This may partly explain the different patterns of molecules produced and secreted in response to an acute bout of exercise compared to an acute inflammatory response to pathogens (as illustrated in figure 5). We further speculate that the increased mRNA expression levels of cytokines in PBMCs may induce a regenerative response in patrolling PBMCs, possibly as an attempt to restore homeostasis [167, 186, 265]. In our studies, we therefore believe that the response observed in PBMCs may provide valuable insight into functions of the immune system, rather than reflecting the responses in skeletal muscle. Thus, the altered response in gene expression levels from both skeletal muscle and PBMCs contribute to the interpretation of the effects observed after acute exercise and strength training in combination with protein supplementation (paper III and IV).

Further, we found that the response of *IL10* in PBMCs was attenuated in older compared to younger adults, whereas mRNA expression levels of *IL8* and *CCL3* were higher in older compared to younger adults after exercise, as illustrated in Figure 9a. The exact physiological explanation of these differences are not known, but may be related to the senescence of the

immune system observed with increasing age. It may also be explained by differences in baseline expression levels between young and older participants. Moreover, biopsies were taken at one time point only, leaving uncertainty about the expression levels before and after this time point. In addition, younger subjects were stronger and lifted approximately twice the volume of the older participants possibly affecting the relative systemic stress (e.g. circulatory system etc.) and potentially the response in PBMCs.

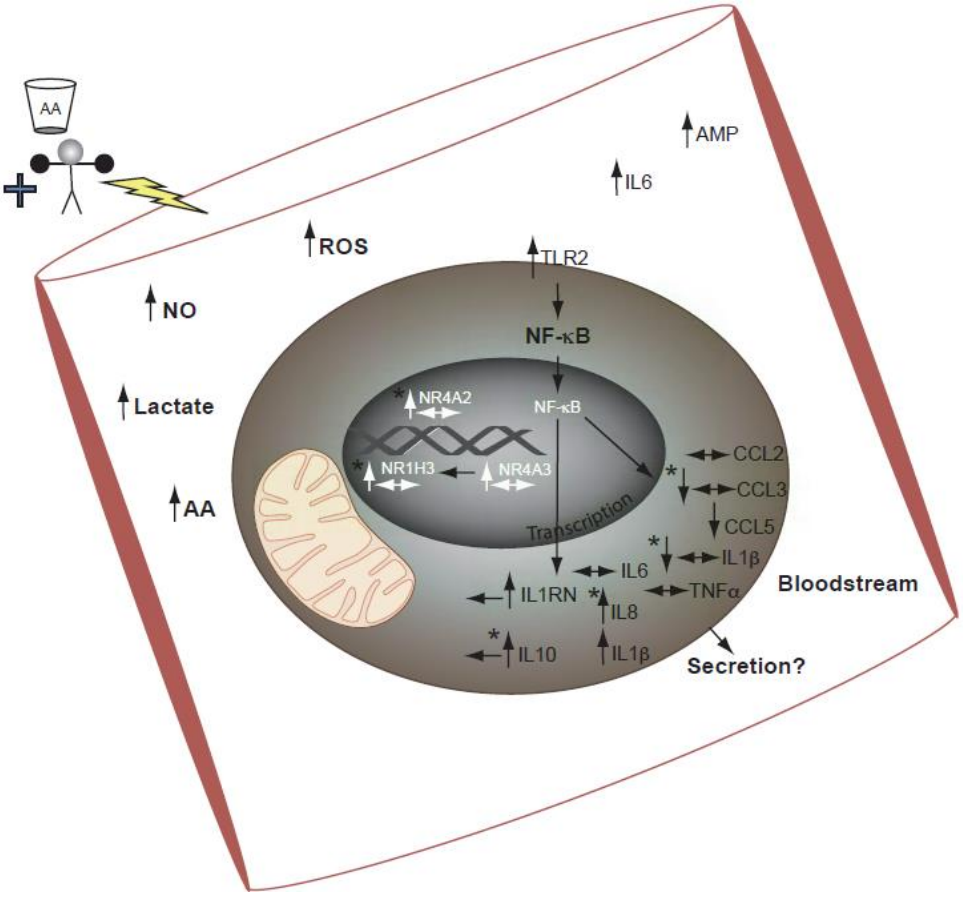


Figure 9a. The acute response to high-load strength exercise in combination with protein supplementation in PBMCs of selected genes. Arrows in front of the gene symbol indicate the direction of the response in young (arrow to the left) and older adults (arrow to the right). When only one arrow is placed in front of the gene symbol, the direction of the response was similar in young and older adults. ↔, no change; ↓, decreased mRNA expression; ↑, increased mRNA expression. * indicate statistically significant differences in the response between young and older. Abbreviations used in figure: AA, amino acids; AMP, AMP-activated protein; CCL, chemokine (C-C motif); IL, interleukin; NO, nitric oxide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NR1H3, Nuclear Receptor Subfamily 1 Group H Member 3; NR4A2/3, Nuclear Receptor Subfamily 4 Group A Member 2/3; ROS, reactive oxygen species; TLR, toll-like receptor; TNF, tumor necrosis factor alpha.

In contrast to others [188, 189, 267, 268], we observed no differences in mRNA expression levels in skeletal muscle between young and older participants after acute exercise, as shown in Figure 9b.

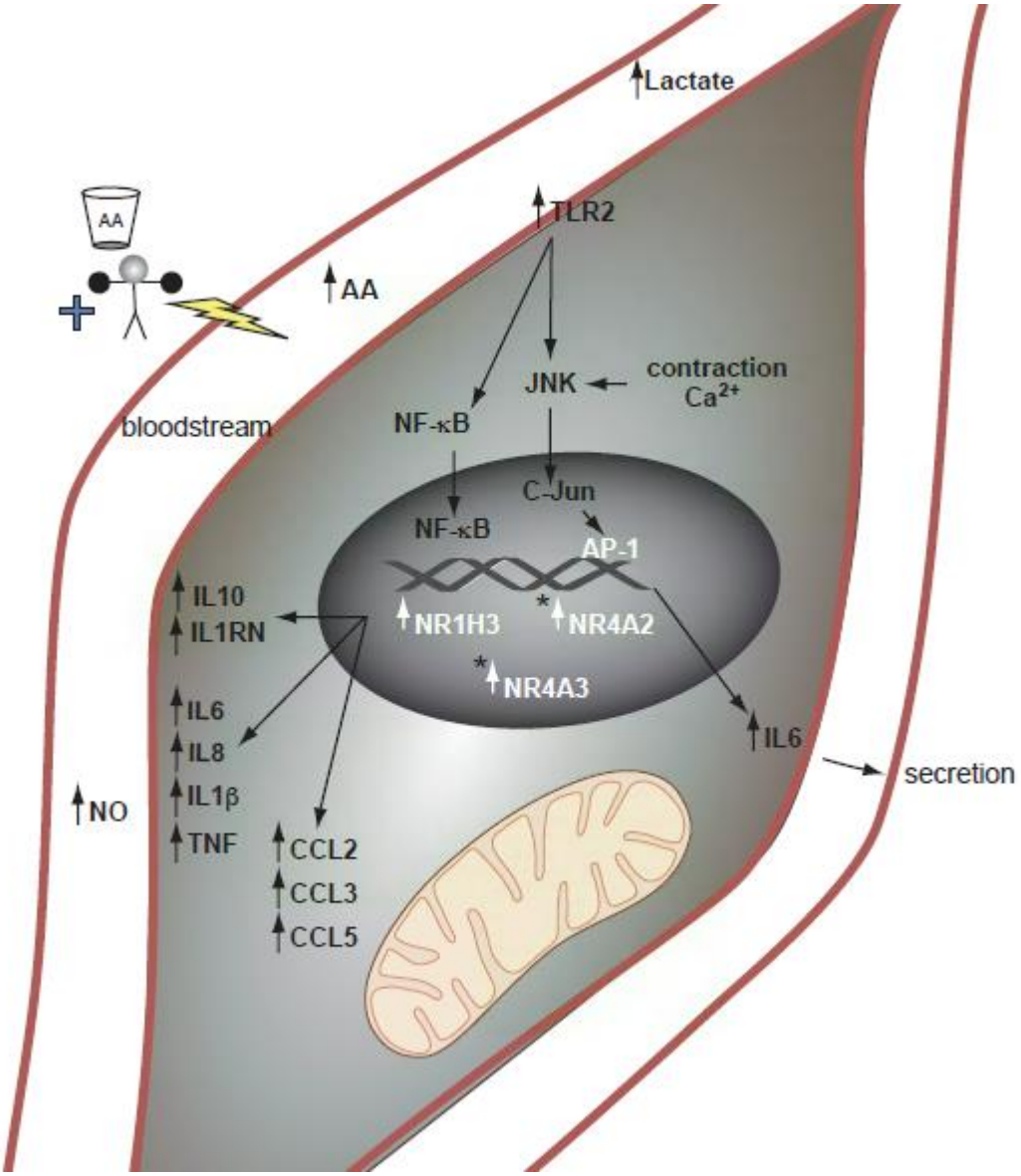


Figure 9b. The acute response to high-load strength exercise in combination with protein supplementation in skeletal muscle of selected genes. One arrow in front of the gene symbol indicates the direction of the response in both young and older adults. ↔, no change; ↓, decreased mRNA expression; ↑, increased mRNA expression. * indicate statistically significant differences in the response between young and older. Abbreviations used in figure: AA, amino acids; AP-1, Activator protein 1; $Ca^{2+,}$ calcium; CCL, chemokine (C-C motif); IL, interleukin; JNK, c-Jun N-terminal kinases; NO, nitric oxide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NR1H3, Nuclear Receptor Subfamily 1 Group H Member 3; NR4A2/3, Nuclear Receptor Subfamily 4 Group A Member 2/3; TLR, toll-like receptor; TNF, tumor necrosis factor alpha.

A possible explanation for the conflicting results may be differences in the exercise session performed. By standardizing the exercise protocol to repetition maximum (RM) sets, as we did in the present study, we ensured that skeletal muscle of both young and old participants were exposed to the same relative intensity, making the relative stress put on the exercising muscles comparable between groups, supporting that young and older muscle adapt similarly to the performed exercise. The altered expression of immune related markers in skeletal muscle may partly originate from immune cells, among them resident macrophages [268], or from muscle-induced production of these molecule mediated by immune cells [189]. One explanation for the lack of differences in cytokine expression in skeletal muscle between young and older participants may therefore be the time points investigated. The biopsies may have been taken too early to detect changes from resident or infiltrating immune cells.

In paper IV we showed that strength training lowered baseline levels of *IL6, IL8* and *CCL3* in PBMCs of older participants after the intervention period, supporting the notion that regular exercise may protect against chronic low-grade inflammation [164]. In contrast, we found a significant upregulation of TLRs in PBMCs as well as skeletal muscle after the training period. Decreased TLR expression has been proposed to be beneficial as it may decrease the inflammatory capacity of leukocytes, possibly promoting an anti-inflammatory milieu in the whole body [269]. TLRs trigger intracellular pathways, among them NF-κB, potentially leading to the induction of inflammatory cytokines including IL1β, IL6, IL8 and TNF [269]. However, we did not observe increased levels of these markers in the present study and TLRs may potentially also activate other pathways, such as p38 mitogen-activated kinase (MAPK) and C-Jun N-terminal kinase (JNK) [270], possibly stimulating cell proliferation [271]. In skeletal muscle, we further observed increased levels of immune-related mRNA transcripts, such as *CCL2, CCL5* and *IL8*. CCL2, CCL5 and IL8 may play important roles in the recruitment of immune-related cells to the skeletal muscle following an acute exercise bout [272, 273], and may be involved in removing cellular debris, releasing factors to promote muscle growth, and facilitating vascular and muscular repair processes [274-276]. The effects, and the mission of these markers after long-term training, are less known. The transient increase of most immune-related markers in skeletal muscle are thought to return to baseline within ~48 hours [277], but it cannot be excluded that some markers will be elevated also after 48 hours. The increased levels of immune related markers observed after the intervention period in the present study could therefore partly be explained by this phenomenon.

The immune modulating effects of training may be multifactorial as regular physical activity can change the distribution of adipose tissue, reduce triglycerides and low-density lipoprotein cholesterol levels and increase high-density lipoprotein [139] and increase skeletal muscle mass and strength [197, 198], amongst other factors. All these factors are also closely linked to inflammation. These may therefore be confounding factors when studying inflammation in relation to exercise training (Kasapis & Thompson, 2005; Gleeson et al., 2011). Nevertheless, we were not able to observe any correlations between changes in android fat mass and changes in mRNA transcripts of *IL6, IL8* and *CCL3* after the training period, indicating that the reduced level of inflammatory markers may occur independent of changes in android fat mass (paper IV).

After the training intervention, we also found changes in mRNA expression levels of genes being important in the regulation of lipid and carbohydrate metabolism, such as *NR1H3, PPARGCIA* and *PPARGC1B* [150, 278-281]. These results were in contrast to others who have found these mRNA transcripts to be upregulated after training [150, 282, 283]. The conflicting results may partly be explained by different adaptation processes to strength and endurance training, but may also an effect of the protein supplements consumed in our studies. However, we were not able to differentiate possible effects of training and supplementation due to the study design. Interestingly, genes like *NR1H3, PPARGC1A* and *PPARGC1B* are also shown to be involved in the regulation of inflammation [284, 285], demonstrating complex connections between metabolism and inflammation [286]. Interactions between these processes may enable the organism to organize and redistribute its energy resources during an escalating immune response [286]. Further, a lower level of PPARGC1A has been related to aging [287, 288] and is also observed in patients with type 2 diabetes [287].

In paper II-IV, we primarily analyzed mRNA expression levels. Several factors may determine whether initial changes in mRNA expression levels will develop into functional proteins, such as posttranscriptional modifications and interactions. Gene expression levels will therefore not always correspond to protein abundance [206], which needs to be taken into consideration when interpreting the results from the present studies.

5.2.3 The role of physical activity in healthy aging

The healthier phenotype observed by regular physical activity may be mediated by several factors [139], where increased muscle mass and strength [289] and a reduced level of pro-inflammatory markers [164] are two of them. Randomized controlled trials have shown that strength exercise provides the best protection against sarcopenia [197, 198]. Strength training should therefore be encouraged in old as well as in young adults. A stronger body will make it easier for older adults to perform daily activities [290], improve quality of life and potentially enabling them to extend the period living at home [291]. However, frequency and exercise load may ultimately determine whether the body responds with favorable adaptations or experiences increased inflammation during training [292]. The intensity and load should therefore be carefully monitored to provide optimal effects in a whole body perspective and to avoid adverse effects such as increased inflammation and injuries. Further, specific motivators and barriers to training may differ with age. In addition to already well-known factors, such as education, gender, psychological and physical well-being, people >75 years are more likely to be motivated by health concerns than those aged 63 to 74 years, and medical problems are more likely to prevent them from training compared with their younger counterparts [293]. Exercise programs, aimed specifically for older adults, should therefore be used.

In summary, endurance as well as strength training should be included in personalized training programs to promote health, especially in the aging population [294-296], as there is strong evidence that exercise attenuates the major hallmarks of aging, including, but not limited to, mitochondrial dysfunction, reduced muscle protein synthesis, anabolic resistance and inflammaging [296]. Even though we were not able to show that the addition of 20g protein twice a day improved muscle mass and strength in older adults, it is important to ensure proper energy and protein intakes in older adults to preserve muscle mass and strength, and to maximize the effects of exercise. Further, strength exercise may reduce mRNA expression levels of immune-related genes in PBMCs.

6 Conclusion

In the present studies, we did not manage to demonstrate that increased intake of dairy protein in older adults positively affected muscle mass, muscle strength, functional performance tests or improved inflammatory status substantially compared to the intake of isocaloric control drinks. Therefore, an additional effect of protein on these factors, beyond the recommended daily intake of 1.0-1.3 g protein/kg body weight, remains uncertain. Further, regular milk protein, WPC80 or native whey protein did not substantially alter the response to exercise after high-load strength exercise or strength training, neither in skeletal muscle nor in PBMCs. On the other hand, high-load strength exercise combined with protein supplementation, showed numerous effects on markers related to muscle regeneration and adaptation both in skeletal muscle and PBMCs. Further, strength training may reduce the level of markers known to be involved in chronic low-grade inflammation in PBMCs, possibly contributing to the anti-inflammatory effects observed by regular training. Rather than reflecting the response in skeletal muscle, gene expression analysis of PBMCs provided valuable insight into the response of the immune system to both acute exercise and strength training combined with protein supplementations in the present studies.

Despite the lack of additional effects of protein in the present studies, a sufficient amount of energy, high-quality protein and regular physical activity should be encouraged to ensure healthy aging [32, 46, 297]. Further, regular exercise should contain both endurance and strength training at an appropriate volume and intensity to profit maximally from the beneficial effects of different training regimes [150, 298].

7 Further perspectives

As the population grows and the proportion of older persons increases, the importance of maintaining a good health will only be more important, both personally and for the society. There are limited resources to build nursing homes and hospitals, forcing older adults to remain living at home. To promote healthy aging and prevent the development of disease, more knowledge about the aging process itself, age-related diseases, and the optimal intake and distribution of protein are needed. Further, acknowledging the complexity of the human metabolism and the diversity of foods, additional knowledge about composition and production of foods, dietary patterns and physical activity on different types of cells and organs, including the interactions among these intergraded systems, including the microbiota, are needed. Technologies to explore both genetic (nutrigenetics) and physiologic responses (nutrigenomics) within the human body, using integration of large data sets and bioinformatics (system biology), should be given high priority. Priority should also be given to research focusing on age-specific preferences, such as food choices and liking, and to research exploring motivational factors for better lifestyle choices. Based on a combination of behavioral research and omics technologies, personalized dietary advices and personalized training protocols could be established to promote optimal health at an individual level, which would also favor the society with reductions in costs related to age- and diet-related treatment of diseases.

8 References

1. WHO, *Diet, nutrition and the prevetion of chronic diseases: report of a Joint WHO/FAO Expert Consulattion.* WHO technical report series, 2003.
2. Lee, I.M., et al., *Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy.* Lancet, 2012. **380**(9838): p. 219-29.
3. Ziegler, C.C. and M.A. Sidani, *Diets for successful aging.* Clin Geriatr Med, 2011. **27**(4): p. 577-89.
4. Mozaffarian, D., *Dietary and Policy Priorities for Cardiovascular Disease, Diabetes, and Obesity: A Comprehensive Review.* Circulation, 2016. **133**(2): p. 187-225.
5. WHO, *Preventing chronic diseases: a vital investment.* 2005.
6. Muller, M. and S. Kersten, *Nutrigenomics: goals and strategies.* Nat Rev Genet, 2003. **4**(4): p. 315-22.
7. Bruhat, A. and P. Fafournoux, *Recent advances on molecular mechanisms involved in amino acid control of gene expression.* Curr Opin Clin Nutr Metab Care, 2001. **4**(5): p. 439-43.
8. Riddle, E.S., M.H. Stipanuk, and A.E. Thalacker-Mercer, *Amino acids in healthy aging skeletal muscle.* Front Biosci (Elite Ed), 2016. **8**: p. 326-50.
9. Wu, G., *Dietary protein intake and human health.* Food Funct, 2016. **7**(3): p. 1251-65.
10. Jousse, C., et al., *Amino acids as regulators of gene expression: molecular mechanisms.* Biochem Biophys Res Commun, 2004. **313**(2): p. 447-52.
11. Kilberg, M.S., et al., *The transcription factor network associated with the amino acid response in mammalian cells.* Adv Nutr, 2012. **3**(3): p. 295-306.
12. Kimball, S.R. and L.S. Jefferson, *Control of protein synthesis by amino acid availability.* Curr Opin Clin Nutr Metab Care, 2002. **5**(1): p. 63-7.
13. Walrand, S. and Y. Boirie, *Optimizing protein intake in aging.* Curr Opin Clin Nutr Metab Care, 2005. **8**(1): p. 89-94.
14. Dardevet, D., et al., *Leucine: a key amino acid in ageing-associated sarcopenia?* Nutr Res Rev, 2003. **16**(1): p. 61-70.
15. Munro, N.C.a.H., *Poteins and amino acids*, in *Modern nutrition in health and disease. 8th edition*, O.J. Shils ME, Shike M, Editor. 1994, Williams & Wilkins. A Waverly Company. p. 3-35.
16. Argiles, J.M., et al., *Skeletal Muscle Regulates Metabolism via Interorgan Crosstalk: Roles in Health and Disease.* J Am Med Dir Assoc, 2016. **17**(9): p. 789-96.
17. Rennie, M.J., et al., *Control of the size of the human muscle mass.* Annu Rev Physiol, 2004. **66**: p. 799-828.
18. Bohe, J., et al., *Human muscle protein synthesis is modulated by extracellular, not intramuscular amino acid availability: a dose-response study.* J Physiol, 2003. **552**(Pt 1): p. 315-24.
19. Rennie, M.J., et al., *Branched-chain amino acids as fuels and anabolic signals in human muscle.* J Nutr, 2006. **136**(1 Suppl): p. 264s-8s.
20. Lancha, A.H., Jr., et al., *Dietary protein supplementation in the elderly for limiting muscle mass loss.* Amino Acids, 2017. **49**(1): p. 33-47.
21. Wolfe, R.R., *Control of muscle protein breakdown: effects of activity and nutritional states.* Int J Sport Nutr Exerc Metab, 2001. **11 Suppl**: p. S164-9.
22. Dideriksen, K., S. Reitelseder, and L. Holm, *Influence of amino acids, dietary protein, and physical activity on muscle mass development in humans.* Nutrients, 2013. **5**(3): p. 852-76.

23. Thalacker-Mercer, A.E., et al., *The skeletal muscle transcript profile reflects accommodative responses to inadequate protein intake in younger and older males.* J Nutr Biochem, 2010. **21**(11): p. 1076-82.
24. Baum, J.I., I.Y. Kim, and R.R. Wolfe, *Protein Consumption and the Elderly: What Is the Optimal Level of Intake?* Nutrients, 2016. **8**(6).
25. Nordic Council of Ministers, *Nordic nutrition recommendations 2012: integrating nutrition and physical activity. 5th edition.* Nord 2014:02. Copenhagen, Denmark, 2014.
26. Bauer, J., et al., *Evidence-based recommendations for optimal dietary protein intake in older people: a position paper from the PROT-AGE Study Group.* J Am Med Dir Assoc, 2013. **14**(8): p. 542-59.
27. Volpi, E., et al., *Is the optimal level of protein intake for older adults greater than the recommended dietary allowance?* J Gerontol A Biol Sci Med Sci, 2013. **68**(6): p. 677-81.
28. Volpi, E., et al., *The Response of Muscle Protein Anabolism to Combined Hyperaminoacidemia and Glucose-Induced Hyperinsulinemia Is Impaired in the Elderly.* The Journal of clinical endocrinology and metabolism, 2000. **85**(12): p. 4481-4490.
29. Guillet, C., et al., *Impaired anabolic response of muscle protein synthesis is associated with S6K1 dysregulation in elderly humans.* Faseb j, 2004. **18**(13): p. 1586-7.
30. Rennie, M.J., *Anabolic resistance: the effects of aging, sexual dimorphism, and immobilization on human muscle protein turnover.* Appl Physiol Nutr Metab, 2009. **34**(3): p. 377-81.
31. Wolfe, R.R., S.L. Miller, and K.B. Miller, *Optimal protein intake in the elderly.* Clin Nutr, 2008. **27**(5): p. 675-84.
32. Deutz, N.E., et al., *Protein intake and exercise for optimal muscle function with aging: recommendations from the ESPEN Expert Group.* Clin Nutr, 2014. **33**(6): p. 929-36.
33. Moore, D.R., et al., *Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men.* J Gerontol A Biol Sci Med Sci, 2015. **70**(1): p. 57-62.
34. Helsedirektoratet, *Norkost 3 En landsomfattende kostholdsundersøkelse blant menn og kvinner i Norge i alderen 18-70 år, 2010-11.* 2012.
35. Vinknes, K.J., et al., *Dietary intake of protein is positively associated with percent body fat in middle-aged and older adults.* J Nutr, 2011. **141**(3): p. 440-6.
36. van Staveren, W.A. and L.C. de Groot, *Evidence-based dietary guidance and the role of dairy products for appropriate nutrition in the elderly.* J Am Coll Nutr, 2011. **30**(5 Suppl 1): p. 429s-37s.
37. Campbell, B., et al., *International Society of Sports Nutrition position stand: protein and exercise.* J Int Soc Sports Nutr, 2007. **4**: p. 8.
38. Hu, F.B., *Protein, body weight, and cardiovascular health.* Am J Clin Nutr, 2005. **82**(1 Suppl): p. 242s-247s.
39. Westerterp-Plantenga, M.S., et al., *Dietary protein, weight loss, and weight maintenance.* Annu Rev Nutr, 2009. **29**: p. 21-41.
40. Pasiakos, S.M., *Metabolic advantages of higher protein diets and benefits of dairy foods on weight management, glycemic regulation, and bone.* J Food Sci, 2015. **80 Suppl 1**: p. A2-7.
41. McLeod, M., et al., *Live strong and prosper: the importance of skeletal muscle strength for healthy ageing.* Biogerontology, 2016. **17**(3): p. 497-510.

42. Metter, E.J., et al., *Skeletal muscle strength as a predictor of all-cause mortality in healthy men.* J Gerontol A Biol Sci Med Sci, 2002. **57**(10): p. B359-65.
43. Arnal, M.A., et al., *Protein pulse feeding improves protein retention in elderly women.* American Journal of Clinical Nutrition, 1999. **69**(6): p. 1202-1208.
44. Kim, I.Y., et al., *Quantity of dietary protein intake, but not pattern of intake, affects net protein balance primarily through differences in protein synthesis in older adults.* Am J Physiol Endocrinol Metab, 2015. **308**(1): p. E21-8.
45. Boirie, Y., et al., *Slow and fast dietary proteins differently modulate postprandial protein accretion.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14930-5.
46. Boirie, Y., et al., *Nutrition and protein energy homeostasis in elderly.* Mech Ageing Dev, 2014. **136-137**: p. 76-84.
47. Schwingshackl, L. and G. Hoffmann, *Comparison of high vs. normal/low protein diets on renal function in subjects without chronic kidney disease: a systematic review and meta-analysis.* PLoS One, 2014. **9**(5): p. e97656.
48. Wolfe, B.M., *Potential role of raising dietary protein intake for reducing risk of atherosclerosis.* Can J Cardiol, 1995. **11 Suppl G**: p. 127g-131g.
49. Santesso, N., et al., *Effects of higher- versus lower-protein diets on health outcomes: a systematic review and meta-analysis.* Eur J Clin Nutr, 2012. **66**(7): p. 780-8.
50. Kitabchi, A.E., et al., *Effects of high-protein versus high-carbohydrate diets on markers of beta-cell function, oxidative stress, lipid peroxidation, proinflammatory cytokines, and adipokines in obese, premenopausal women without diabetes: a randomized controlled trial.* Diabetes Care, 2013. **36**(7): p. 1919-25.
51. Comerford, K.B. and G. Pasin, *Emerging Evidence for the Importance of Dietary Protein Source on Glucoregulatory Markers and Type 2 Diabetes: Different Effects of Dairy, Meat, Fish, Egg, and Plant Protein Foods.* Nutrients, 2016. **8**(8).
52. Helsedirektoratet, *Kostråd for å fremme folkehelsen og forebygge kroniske sykdommer – Metodologi og vitenskapelig kunnskapsgrunnlag.* 2011.
53. Walstra P, W.J., Geurts TJ, *Dairy Science and Technology*. Second ed. 2006: Taylor & Francis Group.
54. Krissansen, G.W., *Emerging health properties of whey proteins and their clinical implications.* J Am Coll Nutr, 2007. **26**(6): p. 713s-23s.
55. Dangin, M., et al., *Influence of the protein digestion rate on protein turnover in young and elderly subjects.* J Nutr, 2002. **132**(10): p. 3228S-33S.
56. Gryson, C., et al., *"Fast proteins" with a unique essential amino acid content as an optimal nutrition in the elderly: Growing evidence.* Clin Nutr, 2013.
57. Smithers, G.W., *Whey and whey proteins—From 'gutter-to-gold'.* International Dairy Journal, 2008. **18**(7): p. 695-704.
58. Sousa, G.T., et al., *Dietary whey protein lessens several risk factors for metabolic diseases: a review.* Lipids Health Dis, 2012. **11**: p. 67.
59. Zemel, M.B., *The role of dairy foods in weight management.* J Am Coll Nutr, 2005. **24**(6 Suppl): p. 537s-46s.
60. Fekete, A.A., D.I. Givens, and J.A. Lovegrove, *The impact of milk proteins and peptides on blood pressure and vascular function: a review of evidence from human intervention studies.* Nutr Res Rev, 2013. **26**(2): p. 177-90.
61. Jakubowicz, D. and O. Froy, *Biochemical and metabolic mechanisms by which dietary whey protein may combat obesity and Type 2 diabetes.* J Nutr Biochem, 2013. **24**(1): p. 1-5.
62. Calder, P.C., et al., *Inflammatory disease processes and interactions with nutrition.* Br J Nutr, 2009. **101 Suppl 1**: p. S1-45.

63. Hamer, M., D. Wolvers, and R. Albers, *Using stress models to evaluate immuno-modulating effects of nutritional intervention in healthy individuals.* J Am Coll Nutr, 2004. **23**(6): p. 637-46.
64. Ponnappan, S. and U. Ponnappan, *Aging and immune function: molecular mechanisms to interventions.* Antioxid Redox Signal, 2011. **14**(8): p. 1551-85.
65. Albers, R., et al., *Monitoring immune modulation by nutrition in the general population: identifying and substantiating effects on human health.* Br J Nutr, 2013. **110 Suppl 2**: p. S1-30.
66. Baylis, D., et al., *Understanding how we age: insights into inflammaging.* Longev Healthspan, 2013. **2**(1): p. 8.
67. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
68. Packard, R.R. and P. Libby, *Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction.* Clin Chem, 2008. **54**(1): p. 24-38.
69. Ford, E.S., *The metabolic syndrome and C-reactive protein, fibrinogen, and leukocyte count: findings from the Third National Health and Nutrition Examination Survey.* Atherosclerosis, 2003. **168**(2): p. 351-8.
70. Tamakoshi, K., et al., *The metabolic syndrome is associated with elevated circulating C-reactive protein in healthy reference range, a systemic low-grade inflammatory state.* Int J Obes Relat Metab Disord, 2003. **27**(4): p. 443-9.
71. Pradhan, A.D., et al., *C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus.* Jama, 2001. **286**(3): p. 327-34.
72. Dandona, P., A. Aljada, and A. Bandyopadhyay, *Inflammation: the link between insulin resistance, obesity and diabetes.* Trends Immunol, 2004. **25**(1): p. 4-7.
73. Pickup, J.C., *Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes.* Diabetes Care, 2004. **27**(3): p. 813-23.
74. Dehghan, A., et al., *Genetic variation, C-reactive protein levels, and incidence of diabetes.* Diabetes, 2007. **56**(3): p. 872-8.
75. Calder, P.C., et al., *Dietary factors and low-grade inflammation in relation to overweight and obesity.* Br J Nutr, 2011. **106 Suppl 3**: p. S5-78.
76. Zhang, J.M. and J. An, *Cytokines, inflammation, and pain.* Int Anesthesiol Clin, 2007. **45**(2): p. 27-37.
77. Di Raimondo, D., et al., *Are the Myokines the Mediators of Physical Activity-Induced Health Benefits?* Curr Pharm Des, 2016. **22**(24): p. 3622-47.
78. Borish, L.C. and J.W. Steinke, *2. Cytokines and chemokines.* Journal of Allergy and Clinical Immunology, 2003. **111**(2, Supplement 2): p. S460-S475.
79. Trayhurn, P., C.A. Drevon, and J. Eckel, *Secreted proteins from adipose tissue and skeletal muscle - adipokines, myokines and adipose/muscle cross-talk.* Arch Physiol Biochem, 2011. **117**(2): p. 47-56.
80. Pedersen, B.K., *Muscle as a secretory organ.* Compr Physiol, 2013. **3**(3): p. 1337-62.
81. Franceschi, C. and J. Campisi, *Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases.* J Gerontol A Biol Sci Med Sci, 2014. **69 Suppl 1**: p. S4-9.
82. Singh, T. and A.B. Newman, *Inflammatory markers in population studies of aging.* Ageing Res Rev, 2011. **10**(3): p. 319-29.
83. Beavers, K.M., et al., *Long-term physical activity and inflammatory biomarkers in older adults.* Med Sci Sports Exerc, 2010. **42**(12): p. 2189-96.
84. Franceschi, C., *Inflammaging as a major characteristic of old people: can it be prevented or cured?* Nutr Rev, 2007. **65**(12 Pt 2): p. S173-6.
85. Cevenini, E., D. Monti, and C. Franceschi, *Inflamm-ageing.* Curr Opin Clin Nutr Metab Care, 2013. **16**(1): p. 14-20.

86. Jensen, G.L., *Inflammation: roles in aging and sarcopenia.* JPEN J Parenter Enteral Nutr, 2008. **32**(6): p. 656-9.
87. Beyer, I., T. Mets, and I. Bautmans, *Chronic low-grade inflammation and age-related sarcopenia.* Curr Opin Clin Nutr Metab Care, 2012. **15**(1): p. 12-22.
88. Cruz-Jentoft, A.J., et al., *Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People.* Age Ageing, 2010. **39**(4): p. 412-23.
89. Aleman, H., et al., *Longitudinal evidence on the association between interleukin-6 and C-reactive protein with the loss of total appendicular skeletal muscle in free-living older men and women.* Age Ageing, 2011. **40**(4): p. 469-75.
90. Visser, M., et al., *Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: the Health ABC Study.* J Gerontol A Biol Sci Med Sci, 2002. **57**(5): p. M326-32.
91. Schaap, L.A., et al., *Inflammatory markers and loss of muscle mass (sarcopenia) and strength.* Am J Med, 2006. **119**(6): p. 526.e9-17.
92. Taaffe, D.R., et al., *Cross-sectional and prospective relationships of interleukin-6 and C-reactive protein with physical performance in elderly persons: MacArthur studies of successful aging.* J Gerontol A Biol Sci Med Sci, 2000. **55**(12): p. M709-15.
93. Toth, M.J., et al., *Age-related differences in skeletal muscle protein synthesis: relation to markers of immune activation.* Am J Physiol Endocrinol Metab, 2005. **288**(5): p. E883-91.
94. Lang, C.H., et al., *TNF-alpha impairs heart and skeletal muscle protein synthesis by altering translation initiation.* Am J Physiol Endocrinol Metab, 2002. **282**(2): p. E336-47.
95. Galland, L., *Diet and inflammation.* Nutr Clin Pract, 2010. **25**(6): p. 634-40.
96. Panagiotakos, D.B., et al., *Dairy products consumption is associated with decreased levels of inflammatory markers related to cardiovascular disease in apparently healthy adults: the ATTICA study.* J Am Coll Nutr, 2010. **29**(4): p. 357-64.
97. Esmaillzadeh, A. and L. Azadbakht, *Dairy consumption and circulating levels of inflammatory markers among Iranian women.* Public Health Nutr, 2010. **13**(9): p. 1395-402.
98. Rashidi Pour Fard, N., et al., *Dairy consumption, cardiovascular risk factors and inflammation in elderly subjects.* ARYA Atheroscler, 2015. **11**(6): p. 323-31.
99. Labonte, M.E., et al., *Dairy Product Consumption Has No Impact on Biomarkers of Inflammation among Men and Women with Low-Grade Systemic Inflammation.* J Nutr, 2014. **144**(11): p. 1760-7.
100. Pal, S. and V. Ellis, *Acute effects of whey protein isolate on blood pressure, vascular function and inflammatory markers in overweight postmenopausal women.* Br J Nutr, 2011. **105**(10): p. 1512-9.
101. Ballard, K.D., et al., *Acute ingestion of a novel whey-derived peptide improves vascular endothelial responses in healthy individuals: a randomized, placebo controlled trial.* Nutr J, 2009. **8**: p. 34.
102. Kerasioti, E., et al., *Anti-inflammatory effects of a special carbohydrate-whey protein cake after exhaustive cycling in humans.* Food Chem Toxicol, 2013. **61**: p. 42-6.
103. Holmer-Jensen, J., et al., *Differential effects of dietary protein sources on postprandial low-grade inflammation after a single high fat meal in obese non-diabetic subjects.* Nutr J, 2011. **10**: p. 115.
104. Pal, S. and V. Ellis, *The chronic effects of whey proteins on blood pressure, vascular function, and inflammatory markers in overweight individuals.* Obesity (Silver Spring), 2010. **18**(7): p. 1354-9.

105. Sugawara, K., et al., *Effect of anti-inflammatory supplementation with whey peptide and exercise therapy in patients with COPD.* Respir Med, 2012. **106**(11): p. 1526-34.
106. Arnberg, K., et al., *Casein improves brachial and central aortic diastolic blood pressure in overweight adolescents: a randomised, controlled trial.* J Nutr Sci, 2013. **2**: p. e43.
107. Ha, E. and M.B. Zemel, *Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people (review).* The Journal of Nutritional Biochemistry, 2003. **14**(5): p. 251-258.
108. Marshall, K., *Therapeutic applications of whey protein.* Altern Med Rev, 2004. **9**(2): p. 136-56.
109. Ward, P.P., E. Paz, and O.M. Conneely, *Multifunctional roles of lactoferrin: a critical overview.* Cell Mol Life Sci, 2005. **62**(22): p. 2540-8.
110. Zemel, M.B., *Proposed role of calcium and dairy food components in weight management and metabolic health.* Phys Sportsmed, 2009. **37**(2): p. 29-39.
111. Madureira, A.R., et al., *Invited review: physiological properties of bioactive peptides obtained from whey proteins.* J Dairy Sci, 2010. **93**(2): p. 437-55.
112. Kratz, M., T. Baars, and S. Guyenet, *The relationship between high-fat dairy consumption and obesity, cardiovascular, and metabolic disease.* Eur J Nutr, 2013. **52**(1): p. 1-24.
113. Da Silva, M.S. and I. Rudkowska, *Dairy nutrients and their effect on inflammatory profile in molecular studies.* Mol Nutr Food Res, 2015. **59**(7): p. 1249-63.
114. Tønnesen M, L.S., Syse A, *Population projections, 2016-2100: Main results.* Translation from Economic Survey 21 june 2016, 2016.
115. WHO, *World report on ageing and health.* 2015.
116. Mitchell, W.K., et al., *Sarcopenia, dynapenia, and the impact of advancing age on human skeletal muscle size and strength; a quantitative review.* Front Physiol, 2012. **3**: p. 260.
117. Lexell, J., *Human aging, muscle mass, and fiber type composition.* J Gerontol A Biol Sci Med Sci, 1995. **50 Spec No**: p. 11-6.
118. Luhrmann, P.M., et al., *Longitudinal changes in energy expenditure in an elderly German population: a 12-year follow-up.* Eur J Clin Nutr, 2009. **63**(8): p. 986-92.
119. Baumgartner, R.N., et al., *Predictors of skeletal muscle mass in elderly men and women.* Mech Ageing Dev, 1999. **107**(2): p. 123-36.
120. Roubenoff, R., *Physical activity, inflammation, and muscle loss.* Nutr Rev, 2007. **65**(12 Pt 2): p. S208-12.
121. da Silva Alexandre, T., et al., *Sarcopenia according to the european working group on sarcopenia in older people (EWGSOP) versus Dynapenia as a risk factor for disability in the elderly.* J Nutr Health Aging, 2014. **18**(5): p. 547-53.
122. Ambrose, A.F., G. Paul, and J.M. Hausdorff, *Risk factors for falls among older adults: a review of the literature.* Maturitas, 2013. **75**(1): p. 51-61.
123. Baumgartner, R.N., et al., *Epidemiology of sarcopenia among the elderly in New Mexico.* Am J Epidemiol, 1998. **147**(8): p. 755-63.
124. Abellan van Kan, G., *Epidemiology and consequences of sarcopenia.* J Nutr Health Aging, 2009. **13**(8): p. 708-12.
125. von Haehling, S., J.E. Morley, and S.D. Anker, *An overview of sarcopenia: facts and numbers on prevalence and clinical impact.* J Cachexia Sarcopenia Muscle, 2010. **1**(2): p. 129-133.
126. Iannuzzi-Sucich, M., K.M. Prestwood, and A.M. Kenny, *Prevalence of sarcopenia and predictors of skeletal muscle mass in healthy, older men and women.* J Gerontol A Biol Sci Med Sci, 2002. **57**(12): p. M772-7.

127. Murton, A.J., *Muscle protein turnover in the elderly and its potential contribution to the development of sarcopenia.* Proc Nutr Soc, 2015. **74**(4): p. 387-96.
128. Houston, D.K., et al., *Dietary protein intake is associated with lean mass change in older, community-dwelling adults: the Health, Aging, and Body Composition (Health ABC) Study.* Am J Clin Nutr, 2008. **87**(1): p. 150-5.
129. Rieu, I., et al., *Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia.* J Physiol, 2006. **575**(Pt 1): p. 305-15.
130. Volpi, E., et al., *Exogenous amino acids stimulate net muscle protein synthesis in the elderly.* J Clin Invest, 1998. **101**(9): p. 2000-7.
131. Volpi, E., et al., *Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults.* Am J Clin Nutr, 2003. **78**(2): p. 250-8.
132. Paddon-Jones, D., et al., *Amino acid ingestion improves muscle protein synthesis in the young and elderly.* Am J Physiol Endocrinol Metab, 2004. **286**(3): p. E321-8.
133. Paddon-Jones, D., et al., *Differential stimulation of muscle protein synthesis in elderly humans following isocaloric ingestion of amino acids or whey protein.* Exp Gerontol, 2006. **41**(2): p. 215-9.
134. Moore, D.R., *Keeping older muscle "young" through dietary protein and physical activity.* Adv Nutr, 2014. **5**(5): p. 599s-607s.
135. Breen, L. and S.M. Phillips, *Skeletal muscle protein metabolism in the elderly: Interventions to counteract the 'anabolic resistance' of ageing.* Nutr Metab (Lond), 2011. **8**: p. 68.
136. Deutz, N.E. and R.R. Wolfe, *Is there a maximal anabolic response to protein intake with a meal?* Clin Nutr, 2013. **32**(2): p. 309-13.
137. Verhoeven, S., et al., *Long-term leucine supplementation does not increase muscle mass or strength in healthy elderly men.* Am J Clin Nutr, 2009. **89**(5): p. 1468-75.
138. Campins, L., et al., *Oral Drugs Related with Muscle Wasting and Sarcopenia. A Review.* Pharmacology, 2017. **99**(1-2): p. 1-8.
139. Warburton, D.E., C.W. Nicol, and S.S. Bredin, *Health benefits of physical activity: the evidence.* Cmaj, 2006. **174**(6): p. 801-9.
140. de Sousa, C.V., et al., *The Antioxidant Effect of Exercise: A Systematic Review and Meta-Analysis.* Sports Med, 2017. **47**(2): p. 277-293.
141. Wilmot, E.G., et al., *Sedentary time in adults and the association with diabetes, cardiovascular disease and death: systematic review and meta-analysis.* Diabetologia, 2012. **55**(11): p. 2895-905.
142. Booth, F.W., et al., *Waging war on physical inactivity: using modern molecular ammunition against an ancient enemy.* J Appl Physiol (1985), 2002. **93**(1): p. 3-30.
143. WHO, *Global Strategy on Diet, Physical Activity and Health.* 2004.
144. Kokkinos, P., et al., *Exercise capacity and mortality in black and white men.* Circulation, 2008. **117**(5): p. 614-22.
145. Helsedirektoratet, *Anbefalinger for fysisk aktivitet.* https://helsenorge.no/SiteCollectionDocuments/Nasjonale%20anbefalinger%2018-64.pdf, 2014.
146. Janssen, I., et al., *Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr.* J Appl Physiol (1985), 2000. **89**(1): p. 81-8.
147. Hoppeler, H., et al., *Molecular mechanisms of muscle plasticity with exercise.* Compr Physiol, 2011. **1**(3): p. 1383-412.
148. Vogt, M., et al., *Effects of dietary fat on muscle substrates, metabolism, and performance in athletes.* Med Sci Sports Exerc, 2003. **35**(6): p. 952-60.

149. Demontis, F., et al., *The influence of skeletal muscle on systemic aging and lifespan.* Aging Cell, 2013. **12**(6): p. 943-9.
150. Egan, B. and J.R. Zierath, *Exercise metabolism and the molecular regulation of skeletal muscle adaptation.* Cell Metab, 2013. **17**(2): p. 162-84.
151. Tipton, K.D. and S.M. Phillips, *Dietary protein for muscle hypertrophy.* Nestle Nutr Inst Workshop Ser, 2013. **76**: p. 73-84.
152. McGlory, C. and S.M. Phillips, *Exercise and the Regulation of Skeletal Muscle Hypertrophy.* Prog Mol Biol Transl Sci, 2015. **135**: p. 153-73.
153. Bassit, R.A., et al., *Branched-chain amino acid supplementation and the immune response of long-distance athletes.* Nutrition, 2002. **18**(5): p. 376-9.
154. Tang, J.E., et al., *Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men.* J Appl Physiol (1985), 2009. **107**(3): p. 987-92.
155. Phillips, S.M., J.E. Tang, and D.R. Moore, *The role of milk- and soy-based protein in support of muscle protein synthesis and muscle protein accretion in young and elderly persons.* J Am Coll Nutr, 2009. **28**(4): p. 343-54.
156. Burd, N.A., et al., *Greater stimulation of myofibrillar protein synthesis with ingestion of whey protein isolate v. micellar casein at rest and after resistance exercise in elderly men.* Br J Nutr, 2012. **108**(6): p. 958-62.
157. Pennings, B., et al., *Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men.* Am J Clin Nutr, 2011. **93**(5): p. 997-1005.
158. Pedersen, B.K. and M.A. Febbraio, *Muscle as an endocrine organ: focus on muscle-derived interleukin-6.* Physiol Rev, 2008. **88**(4): p. 1379-406.
159. Norheim, F., et al., *Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training.* Am J Physiol Endocrinol Metab, 2011. **301**(5): p. E1013-21.
160. Eckardt, K., et al., *Myokines in insulin resistance and type 2 diabetes.* Diabetologia, 2014. **57**(6): p. 1087-99.
161. Pedersen, B.K. and C.P. Fischer, *Beneficial health effects of exercise--the role of IL-6 as a myokine.* Trends Pharmacol Sci, 2007. **28**(4): p. 152-6.
162. Teixeira-Lemos, E., et al., *Regular physical exercise training assists in preventing type 2 diabetes development: focus on its antioxidant and anti-inflammatory properties.* Cardiovasc Diabetol, 2011. **10**: p. 12.
163. Cornelissen, V.A. and N.A. Smart, *Exercise training for blood pressure: a systematic review and meta-analysis.* J Am Heart Assoc, 2013. **2**(1): p. e004473.
164. Gleeson, M., et al., *The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease.* Nat Rev Immunol, 2011. **11**(9): p. 607-15.
165. Nimmo, M.A., et al., *The effect of physical activity on mediators of inflammation.* Diabetes Obes Metab, 2013. **15 Suppl 3**: p. 51-60.
166. Kasapis, C. and P.D. Thompson, *The effects of physical activity on serum C-reactive protein and inflammatory markers: a systematic review.* J Am Coll Cardiol, 2005. **45**(10): p. 1563-9.
167. Cruzat, V.F., M. Krause, and P. Newsholme, *Amino acid supplementation and impact on immune function in the context of exercise.* J Int Soc Sports Nutr, 2014. **11**(1): p. 61.
168. Febbraio, M.A., et al., *Glucose ingestion attenuates interleukin-6 release from contracting skeletal muscle in humans.* J Physiol, 2003. **549**(Pt 2): p. 607-12.

169. Spiering, B.A., et al., *Resistance exercise biology: manipulation of resistance exercise programme variables determines the responses of cellular and molecular signalling pathways.* Sports Med, 2008. **38**(7): p. 527-40.
170. Jensen, J., et al., *The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise.* Front Physiol, 2011. **2**: p. 112.
171. Biolo, G., et al., *Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans.* Am J Physiol, 1995. **268**(3 Pt 1): p. E514-20.
172. Damas, F., et al., *A review of resistance training-induced changes in skeletal muscle protein synthesis and their contribution to hypertrophy.* Sports Med, 2015. **45**(6): p. 801-7.
173. Calle, M.C. and M.L. Fernandez, *Effects of resistance training on the inflammatory response.* Nutr Res Pract, 2010. **4**(4): p. 259-69.
174. Chorell, E., et al., *Predictive metabolomics evaluation of nutrition-modulated metabolic stress responses in human blood serum during the early recovery phase of strenuous physical exercise.* J Proteome Res, 2009. **8**(6): p. 2966-77.
175. Pillon, N.J., et al., *Cross-talk between skeletal muscle and immune cells: muscle-derived mediators and metabolic implications.* Am J Physiol Endocrinol Metab, 2013. **304**(5): p. E453-65.
176. Leung, F.P., et al., *Exercise, vascular wall and cardiovascular diseases: an update (Part 1).* Sports Med, 2008. **38**(12): p. 1009-24.
177. Hansson, G.K. and A. Hermansson, *The immune system in atherosclerosis.* Nat Immunol, 2011. **12**(3): p. 204-12.
178. Welch, A.A., *Nutritional influences on age-related skeletal muscle loss.* Proc Nutr Soc, 2014. **73**(1): p. 16-33.
179. Bruun, J.M., et al., *Association between measures of insulin sensitivity and circulating levels of interleukin-8, interleukin-6 and tumor necrosis factor-alpha. Effect of weight loss in obese men.* Eur J Endocrinol, 2003. **148**(5): p. 535-42.
180. Young, J.L., P. Libby, and U. Schonbeck, *Cytokines in the pathogenesis of atherosclerosis.* Thromb Haemost, 2002. **88**(4): p. 554-67.
181. Pedersen, B.K., A. Steensberg, and P. Schjerling, *Muscle-derived interleukin-6: possible biological effects.* J Physiol, 2001. **536**(Pt 2): p. 329-37.
182. Petersen, A.M. and B.K. Pedersen, *The anti-inflammatory effect of exercise.* J Appl Physiol (1985), 2005. **98**(4): p. 1154-62.
183. Suzuki, K., et al., *Systemic inflammatory response to exhaustive exercise. Cytokine kinetics.* Exerc Immunol Rev, 2002. **8**: p. 6-48.
184. Mathur, N. and B.K. Pedersen, *Exercise as a mean to control low-grade systemic inflammation.* Mediators Inflamm, 2008. **2008**: p. 109502.
185. Serrano, A.L., et al., *Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy.* Cell Metab, 2008. **7**(1): p. 33-44.
186. Chazaud, B., *Inflammation during skeletal muscle regeneration and tissue remodeling: application to exercise-induced muscle damage management.* Immunol Cell Biol, 2016. **94**(2): p. 140-5.
187. Fry, C.S., et al., *Aging impairs contraction-induced human skeletal muscle mTORC1 signaling and protein synthesis.* Skelet Muscle, 2011. **1**(1): p. 11.
188. Jozsi, A.C., et al., *Aged human muscle demonstrates an altered gene expression profile consistent with an impaired response to exercise.* Mech Ageing Dev, 2000. **120**(1-3): p. 45-56.
189. Hamada, K., et al., *Senescence of human skeletal muscle impairs the local inflammatory cytokine response to acute eccentric exercise.* FASEB J, 2005. **19**(2): p. 264-6.

190. Merritt, E.K., et al., *Heightened muscle inflammation susceptibility may impair regenerative capacity in aging humans.* J Appl Physiol (1985), 2013. **115**(6): p. 937-48.
191. Gano, L.B., et al., *Increased proinflammatory and oxidant gene expression in circulating mononuclear cells in older adults: amelioration by habitual exercise.* Physiol Genomics, 2011. **43**(14): p. 895-902.
192. Ivey, F.M., et al., *Effects of age, gender, and myostatin genotype on the hypertrophic response to heavy resistance strength training.* J Gerontol A Biol Sci Med Sci, 2000. **55**(11): p. M641-8.
193. Ribeiro, A.S., et al., *Effects of traditional and pyramidal resistance training systems on muscular strength, muscle mass, and hormonal responses in older women: a randomized crossover trial.* J Strength Cond Res, 2016.
194. Dirks, M.L., et al., *One Week of Bed Rest Leads to Substantial Muscle Atrophy and Induces Whole-Body Insulin Resistance in the Absence of Skeletal Muscle Lipid Accumulation.* Diabetes, 2016. **65**(10): p. 2862-75.
195. Melov, S., et al., *Resistance exercise reverses aging in human skeletal muscle.* PLoS One, 2007. **2**(5): p. e465.
196. Dideriksen, K.J., et al., *Stimulation of muscle protein synthesis by whey and caseinate ingestion after resistance exercise in elderly individuals.* Scand J Med Sci Sports, 2011. **21**(6): p. e372-83.
197. Law, T.D., L.A. Clark, and B.C. Clark, *Resistance Exercise to Prevent and Manage Sarcopenia and Dynapenia.* Annu Rev Gerontol Geriatr, 2016. **36**(1): p. 205-228.
198. Bibas, L., et al., *Therapeutic interventions for frail elderly patients: part I. Published randomized trials.* Prog Cardiovasc Dis, 2014. **57**(2): p. 134-43.
199. Biolo, G., et al., *An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein.* Am J Physiol, 1997. **273**(1 Pt 1): p. E122-9.
200. Tipton, K.D., et al., *Stimulation of muscle anabolism by resistance exercise and ingestion of leucine plus protein.* Appl Physiol Nutr Metab, 2009. **34**(2): p. 151-61.
201. Williams, R.a.N., PD, *Regulation of Gene Expression in Skeletal Muscle by Contractile Activity.* Compr Physiol 2011, Supplement 29: Handbook of Physiology, Exercise: Regulation and Integration of Multiple Systems: 1124-1150. First published in print 1996., 1996.
202. Egan, B., J.A. Hawley, and J.R. Zierath, *SnapShot: Exercise Metabolism.* Cell Metab, 2016. **24**(2): p. 342-342.e1.
203. Bautmans, I., et al., *Biochemical changes in response to intensive resistance exercise training in the elderly.* Gerontology, 2005. **51**(4): p. 253-65.
204. Ogawa, K., et al., *Resistance exercise training-induced muscle hypertrophy was associated with reduction of inflammatory markers in elderly women.* Mediators Inflamm, 2010. **2010**: p. 171023.
205. Carlberg, U.a.M., *Nutrigenomics*. Vol. 1. 2016: Springer.
206. Norheim, F., et al., *Molecular nutrition research: the modern way of performing nutritional science.* Nutrients, 2012. **4**(12): p. 1898-944.
207. de Mello, V.D., et al., *Gene expression of peripheral blood mononuclear cells as a tool in dietary intervention studies: What do we know so far?* Mol Nutr Food Res, 2012. **56**(7): p. 1160-72.
208. Bouwens, M., L.A. Afman, and M. Muller, *Fasting induces changes in peripheral blood mononuclear cell gene expression profiles related to increases in fatty acid beta-oxidation: functional role of peroxisome proliferator activated receptor alpha in human peripheral blood mononuclear cells.* Am J Clin Nutr, 2007. **86**(5): p. 1515-23.

209. Liew, C.C., et al., *The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool.* J Lab Clin Med, 2006. **147**(3): p. 126-32.
210. Rudkowska, I., et al., *Validation of the use of peripheral blood mononuclear cells as surrogate model for skeletal muscle tissue in nutrigenomic studies.* Omics, 2011. **15**(1-2): p. 1-7.
211. Yang, Y., et al., *Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men.* Br J Nutr, 2012. **108**(10): p. 1780-8.
212. Pennings, B., et al., *Amino acid absorption and subsequent muscle protein accretion following graded intakes of whey protein in elderly men.* Am J Physiol Endocrinol Metab, 2012. **302**(8): p. E992-9.
213. Sibbald, B. and M. Roland, *Understanding controlled trials. Why are randomised controlled trials important?* Bmj, 1998. **316**(7126): p. 201.
214. Hickson, M., *Nutritional interventions in sarcopenia: a critical review.* Proc Nutr Soc, 2015. **74**(4): p. 378-86.
215. Myhrstad, M.C., et al., *Effect of the fat composition of a single high-fat meal on inflammatory markers in healthy young women.* Br J Nutr, 2011. **106**(12): p. 1826-35.
216. Moore, D.R., et al., *Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men.* Am J Clin Nutr, 2009. **89**(1): p. 161-8.
217. Shad, B.J., J.L. Thompson, and L. Breen, *Does the muscle protein synthetic response to exercise and amino acid-based nutrition diminish with advancing age? A systematic review.* Am J Physiol Endocrinol Metab, 2016. **311**(5): p. E803-e817.
218. Tieland, M., et al., *Dietary protein intake in community-dwelling, frail, and institutionalized elderly people: scope for improvement.* Eur J Nutr, 2012. **51**(2): p. 173-9.
219. Paddon-Jones, D. and B.B. Rasmussen, *Dietary protein recommendations and the prevention of sarcopenia.* Curr Opin Clin Nutr Metab Care, 2009. **12**(1): p. 86-90.
220. Mamerow, M.M., et al., *Dietary protein distribution positively influences 24-h muscle protein synthesis in healthy adults.* J Nutr, 2014. **144**(6): p. 876-80.
221. Da Silva, M.S. and I. Rudkowska, *Dairy products on metabolic health: current research and clinical implications.* Maturitas, 2014. **77**(3): p. 221-8.
222. Labonte, M.E., et al., *Impact of dairy products on biomarkers of inflammation: a systematic review of randomized controlled nutritional intervention studies in overweight and obese adults.* Am J Clin Nutr, 2013. **97**(4): p. 706-17.
223. Gjevestad, G.O., K.B. Holven, and S.M. Ulven, *Effects of Exercise on Gene Expression of Inflammatory Markers in Human Peripheral Blood Cells: A Systematic Review.* Curr Cardiovasc Risk Rep, 2015. **9**(7): p. 34.
224. Paulsen, G., et al., *Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise?* Exerc Immunol Rev, 2012. **18**: p. 42-97.
225. Bonizzi, G. and M. Karin, *The two NF-kappaB activation pathways and their role in innate and adaptive immunity.* Trends Immunol, 2004. **25**(6): p. 280-8.
226. Tornatore, L., et al., *The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation.* Trends Cell Biol, 2012. **22**(11): p. 557-66.
227. Teitelbaum, S.L. and F.P. Ross, *Genetic regulation of osteoclast development and function.* Nat Rev Genet, 2003. **4**(8): p. 638-49.
228. Arnold, M.A., et al., *MEF2C transcription factor controls chondrocyte hypertrophy and bone development.* Dev Cell, 2007. **12**(3): p. 377-89.

229. Barns, M., et al., *Molecular analyses provide insight into mechanisms underlying sarcopenia and myofibre denervation in old skeletal muscles of mice.* Int J Biochem Cell Biol, 2014. **53**: p. 174-85.
230. Zhong, J., X. Rao, and S. Rajagopalan, *An emerging role of dipeptidyl peptidase 4 (DPP4) beyond glucose control: potential implications in cardiovascular disease.* Atherosclerosis, 2013. **226**(2): p. 305-14.
231. Sakagami, H., et al., *Loss of HIF-1alpha impairs GLUT4 translocation and glucose uptake by the skeletal muscle cells.* Am J Physiol Endocrinol Metab, 2014. **306**(9): p. E1065-76.
232. Bodine, S.C. and L.M. Baehr, *Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1.* Am J Physiol Endocrinol Metab, 2014. **307**(6): p. E469-84.
233. Mohamad, M.I. and M.S. Khater, *Evaluation of insulin like growth factor-1 (IGF-1) level and its impact on muscle and bone mineral density in frail elderly male.* Archives of Gerontology and Geriatrics, 2015. **60**(1): p. 124-127.
234. O'Grada, C.M., et al., *PBMCs reflect the immune component of the WAT transcriptome--implications as biomarkers of metabolic health in the postprandial state.* Mol Nutr Food Res, 2014. **58**(4): p. 808-20.
235. Pasterkamp, G. and M. Daemen, *Circulating cells: the biofactory for markers of atherosclerotic disease.* Eur Heart J, 2008. **29**(22): p. 2701-2.
236. McHale, C.M., et al., *Changes in the peripheral blood transcriptome associated with occupational benzene exposure identified by cross-comparison on two microarray platforms.* Genomics, 2009. **93**(4): p. 343-9.
237. Ulven, S.M., et al., *An acute bout of exercise modulate the inflammatory response in peripheral blood mononuclear cells in healthy young men.* Arch Physiol Biochem, 2015. **121**(2): p. 41-9.
238. Storey, A.G., et al., *Stress responses to short-term intensified and reduced training in competitive weightlifters.* Scand J Med Sci Sports, 2016. **26**(1): p. 29-40.
239. Louis, E., et al., *Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle.* J Appl Physiol (1985), 2007. **103**(5): p. 1744-51.
240. Della Gatta, P.A., D. Cameron-Smith, and J.M. Peake, *Acute resistance exercise increases the expression of chemotactic factors within skeletal muscle.* Eur J Appl Physiol, 2014. **114**(10): p. 2157-67.
241. Ispoglou, T., et al., *Double-blind, placebo-controlled pilot trial of L-Leucine-enriched amino-acid mixtures on body composition and physical performance in men and women aged 65-75 years.* Eur J Clin Nutr, 2016. **70**(2): p. 182-8.
242. Leenders, M., et al., *Prolonged leucine supplementation does not augment muscle mass or affect glycemic control in elderly type 2 diabetic men.* J Nutr, 2011. **141**(6): p. 1070-6.
243. Tieland, M., et al., *Protein supplementation improves physical performance in frail elderly people: a randomized, double-blind, placebo-controlled trial.* J Am Med Dir Assoc, 2012. **13**(8): p. 720-6.
244. Norton, C., et al., *Protein Supplementation at Breakfast and Lunch for 24 Weeks beyond Habitual Intakes Increases Whole-Body Lean Tissue Mass in Healthy Older Adults.* J Nutr, 2016. **146**(1): p. 65-9.
245. Cuthbertson, D., et al., *Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle.* Faseb j, 2005. **19**(3): p. 422-4.
246. Wolfe, R.R., *Regulation of muscle protein by amino acids.* J Nutr, 2002. **132**(10): p. 3219s-24s.

247. Katsanos, C.S., et al., *A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly.* Am J Physiol Endocrinol Metab, 2006. **291**(2): p. E381-7.
248. Bouillanne, O., et al., *Impact of protein pulse feeding on lean mass in malnourished and at-risk hospitalized elderly patients: a randomized controlled trial.* Clin Nutr, 2013. **32**(2): p. 186-92.
249. Gibson, N.R., et al., *Influences of dietary energy and protein on leucine kinetics during feeding in healthy adults.* American Journal of Physiology - Endocrinology and Metabolism, 1996. **270**(2 33-2): p. E282-E291.
250. Schaap, L.A., et al., *Higher inflammatory marker levels in older persons: associations with 5-year change in muscle mass and muscle strength.* J Gerontol A Biol Sci Med Sci, 2009. **64**(11): p. 1183-9.
251. Stancliffe, R.A., T. Thorpe, and M.B. Zemel, *Dairy attentuates oxidative and inflammatory stress in metabolic syndrome.* Am J Clin Nutr, 2011. **94**(2): p. 422-30.
252. Spann, N.J. and C.K. Glass, *Sterols and oxysterols in immune cell function.* Nat Immunol, 2013. **14**(9): p. 893-900.
253. Calkin, A.C. and P. Tontonoz, *Liver x receptor signaling pathways and atherosclerosis.* Arterioscler Thromb Vasc Biol, 2010. **30**(8): p. 1513-8.
254. Schoenborn, J.R. and C.B. Wilson, *Regulation of interferon-gamma during innate and adaptive immune responses.* Adv Immunol, 2007. **96**: p. 41-101.
255. Rosenthal, J., A. Angel, and J. Farkas, *Metabolic fate of leucine: a significant sterol precursor in adipose tissue and muscle.* Am J Physiol, 1974. **226**(2): p. 411-8.
256. Strandberg, E., et al., *Influence of combined resistance training and healthy diet on muscle mass in healthy elderly women: a randomized controlled trial.* J Appl Physiol (1985), 2015. **119**(8): p. 918-25.
257. Dangin, M., et al., *The rate of protein digestion affects protein gain differently during aging in humans.* J Physiol, 2003. **549**(Pt 2): p. 635-44.
258. Candow, D.G., et al., *Effect of whey and soy protein supplementation combined with resistance training in young adults.* Int J Sport Nutr Exerc Metab, 2006. **16**(3): p. 233-44.
259. Palomino, D.C. and L.C. Marti, *Chemokines and immunity.* Einstein (Sao Paulo), 2015. **13**(3): p. 469-73.
260. Pedersen, B.K., et al., *Muscle-derived interleukin-6: lipolytic, anti-inflammatory and immune regulatory effects.* Pflugers Arch, 2003. **446**(1): p. 9-16.
261. Glund, S. and A. Krook, *Role of interleukin-6 signalling in glucose and lipid metabolism.* Acta Physiol (Oxf), 2008. **192**(1): p. 37-48.
262. Lambernd, S., et al., *Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways.* Diabetologia, 2012. **55**(4): p. 1128-1139.
263. Otis, J.S., et al., *Pro-inflammatory mediation of myoblast proliferation.* PLoS One, 2014. **9**(3): p. e92363.
264. Langen, R.C., et al., *Inflammatory cytokines inhibit myogenic differentiation through activation of nuclear factor-kappaB.* Faseb j, 2001. **15**(7): p. 1169-80.
265. Lancaster, G.I. and M.A. Febbraio, *The immunomodulating role of exercise in metabolic disease.* Trends Immunol, 2014. **35**(6): p. 262-9.
266. Whitham, M., et al., *Contraction-induced interleukin-6 gene transcription in skeletal muscle is regulated by c-Jun terminal kinase/activator protein-1.* J Biol Chem, 2012. **287**(14): p. 10771-9.
267. Jozsi, A.C., et al., *Molecular characteristics of aged muscle reflect an altered ability to respond to exercise.* Int J Sport Nutr Exerc Metab, 2001. **11 Suppl**: p. S9-15.

268. Przybyla, B., et al., *Aging alters macrophage properties in human skeletal muscle both at rest and in response to acute resistance exercise.* Exp Gerontol, 2006. **41**(3): p. 320-7.
269. Gleeson, M., B. McFarlin, and M. Flynn, *Exercise and Toll-like receptors.* Exerc Immunol Rev, 2006. **12**: p. 34-53.
270. O'Neill, L.A.J., D. Golenbock, and A.G. Bowie, *The history of Toll-like receptors [mdash] redefining innate immunity.* Nat Rev Immunol, 2013. **13**(6): p. 453-460.
271. Zhang, W. and H.T. Liu, *MAPK signal pathways in the regulation of cell proliferation in mammalian cells.* Cell Res, 2002. **12**(1): p. 9-18.
272. Mathers, J.L., et al., *Early inflammatory and myogenic responses to resistance exercise in the elderly.* Muscle Nerve, 2012. **46**(3): p. 407-12.
273. Vella, L., et al., *Resistance exercise increases NF-kappaB activity in human skeletal muscle.* Am J Physiol Regul Integr Comp Physiol, 2012. **302**(6): p. R667-73.
274. Nielsen, A.R. and B.K. Pedersen, *The biological roles of exercise-induced cytokines: IL-6, IL-8, and IL-15.* Appl Physiol Nutr Metab, 2007. **32**(5): p. 833-9.
275. Fatouros, I.G., et al., *Time-course of changes in oxidative stress and antioxidant status responses following a soccer game.* Journal of strength and conditioning research / National Strength & Conditioning Association, 2010. **24**(12): p. 3278-3286.
276. Smith, C., et al., *The inflammatory response to skeletal muscle injury: illuminating complexities.* Sports Med, 2008. **38**(11): p. 947-69.
277. Mahoney, D.J., et al., *Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise.* Faseb j, 2005. **19**(11): p. 1498-500.
278. Finck, B.N. and D.P. Kelly, *PGC-1 coactivators: inducible regulators of energy metabolism in health and disease.* J Clin Invest, 2006. **116**(3): p. 615-22.
279. Pearen, M.A. and G.E. Muscat, *Minireview: Nuclear hormone receptor 4A signaling: implications for metabolic disease.* Mol Endocrinol, 2010. **24**(10): p. 1891-903.
280. Pearen, M.A. and G.E. Muscat, *Orphan nuclear receptors and the regulation of nutrient metabolism: understanding obesity.* Physiology (Bethesda), 2012. **27**(3): p. 156-66.
281. Paillasse, M.R. and P. de Medina, *The NR4A nuclear receptors as potential targets for anti-aging interventions.* Med Hypotheses, 2015. **84**(2): p. 135-40.
282. Fyfe, J.J., D.J. Bishop, and N.K. Stepto, *Interference between concurrent resistance and endurance exercise: molecular bases and the role of individual training variables.* Sports Med, 2014. **44**(6): p. 743-62.
283. Vissing, K. and P. Schjerling, *Simplified data access on human skeletal muscle transcriptome responses to differentiated exercise.* Sci Data, 2014. **1**: p. 140041.
284. Hamers, A.A., et al., *NR4A nuclear receptors in immunity and atherosclerosis.* Curr Opin Lipidol, 2013. **24**(5): p. 381-5.
285. Handschin, C. and B.M. Spiegelman, *The role of exercise and PGC1alpha in inflammation and chronic disease.* Nature, 2008. **454**(7203): p. 463-9.
286. Hotamisligil, G.S., *Inflammation and metabolic disorders.* Nature, 2006. **444**(7121): p. 860-7.
287. Ling, C., et al., *Multiple environmental and genetic factors influence skeletal muscle PGC-1alpha and PGC-1beta gene expression in twins.* J Clin Invest, 2004. **114**(10): p. 1518-26.
288. Olesen, J., K. Kiilerich, and H. Pilegaard, *PGC-1alpha-mediated adaptations in skeletal muscle.* Pflugers Arch, 2010. **460**(1): p. 153-62.
289. Bauman, A., et al., *Updating the Evidence for Physical Activity: Summative Reviews of the Epidemiological Evidence, Prevalence, and Interventions to Promote "Active Aging".* Gerontologist, 2016. **56 Suppl 2**: p. S268-80.

290. Vina, J., et al., *Biology of frailty: Modulation of ageing genes and its importance to prevent age-associated loss of function.* Mol Aspects Med, 2016. **50**: p. 88-108.
291. Das, P. and R. Horton, *Physical activity-time to take it seriously and regularly.* Lancet, 2016. **388**(10051): p. 1254-5.
292. Kramer, H.F. and L.J. Goodyear, *Exercise, MAPK, and NF-kappaB signaling in skeletal muscle.* J Appl Physiol (1985), 2007. **103**(1): p. 388-95.
293. Newson, R.S. and E.B. Kemps, *Factors that promote and prevent exercise engagement in older adults.* J Aging Health, 2007. **19**(3): p. 470-81.
294. Nair, K.S., *Aging muscle.* Am J Clin Nutr, 2005. **81**(5): p. 953-63.
295. Gulsvik, A.K., et al., *Ageing, physical activity and mortality--a 42-year follow-up study.* Int J Epidemiol, 2012. **41**(2): p. 521-30.
296. Garatachea, N., et al., *Exercise attenuates the major hallmarks of aging.* Rejuvenation Res, 2015. **18**(1): p. 57-89.
297. Cartee, G.D., et al., *Exercise Promotes Healthy Aging of Skeletal Muscle.* Cell Metab, 2016. **23**(6): p. 1034-47.
298. Makanae, Y. and S. Fujita, *Role of Exercise and Nutrition in the Prevention of Sarcopenia.* J Nutr Sci Vitaminol (Tokyo), 2015. **61 Suppl**: p. S125-7.

Paper I

Inger Ottestad, Amund Tjellaug Løvstad, Gyrd Omholt Gjevestad, Håvard Hamarsland, Jūratė Šaltytė Benth, Lene Frost Andersen, Asta Bye, Anne Sofie Biong, Kjetil Retterstøl, Per Ole Iversen, Truls Raastad, Stine M Ulven, Kirsten B Holven.

Intake of a protein-enriched milk and effects on muscle mass and strength. A 12-week randomized placebo controlled trial among community-dwelling older adults. J Nutr Health Aging (2016). doi:10.1007/s12603-016-0856-1

Paper II

Gyrd O. Gjevestad, Inger Ottestad, Anne Sofie Biong, Per Ole Iversen, Kjetil Retterstøl, Truls Raastad, Bjørn S. Skålhegg, Stine M. Ulven and Kirsten B. Holven.

Consumption of protein-enriched milk has minor effects on inflammation in older adults - a 12-week double-blind randomized controlled trial. In press, Mechanisms of Ageing and Development.
http://dx.doi.org/10.1016/j.mad.2017.01.011

Paper III

Gyrd O. Gjevestad, Håvard Hamarsland, Truls Raastad, Inger Ottestad, Jacob J. Christensen, Kristin Eckardt, Christian A. Drevon, Anne S. Biong, Stine M. Ulven and Kirsten B. Holven.

Gene expression is differentially regulated in skeletal muscle and circulating immune cells in response to an acute bout of high-load strength exercise. In press, Genes & Nutrition.

Gene expression is differentially regulated in skeletal muscle and circulating immune cells in response to an acute bout of high-load strength exercise

Gyrd O. Gjevestad[1,2], Håvard Hamarsland[3], Truls Raastad[3], Inger Ottestad[1], Jacob J. Christensen[1,4], Kristin Eckardt[1], Christian A. Drevon[1], Anne S. Biong[2], Stine M. Ulven[1] and Kirsten B. Holven[1,5]

[1] Department of Nutrition, Institute of Basic Medical Sciences, Faculty of Medicine, P.O. Box 1046, Blindern, 0317 University of Oslo, Norway

[2] TINE SA, Centre for Research and Development, P.O. Box 7, Kalbakken, 0902 Oslo, Norway

[3] Department of Physical Performance, Norwegian School of Sport Sciences, P.B. 4104 U.S., 0806 Oslo, Norway

[4] The Lipid Clinic, Oslo University Hospital Rikshospitalet, P.O. Box 4950 Nydalen, 0424 Oslo, Norway

[5] National Advisory Unit on Familial Hypercholesterolemia, Department of Endocrinology, Morbid Obesity and Preventive Medicine, Oslo University Hospital, P.O. Box 4950 Nydalen, 0424 Oslo, Norway

E-mail addresses:

Gyrd O. Gjevestad; g.o.gjevestad@medisin.uio.no,

Håvard Hamarsland; havard.hamarsland@nih.no

Truls Raastad; truls.raastad@nih.no

Inger Ottestad; inger.ottestad@medisin.uio.no,

Jacob J. Christensen; j.j.christensen@medisin.uio.no

Kristin Eckardt; kristin.eckardt@medisin.uio.no

Christian A. Drevon; c.a.drevon@medisin.uio.no

Anne S. Biong; anne.sofie.biong@tine.no,

Stine M. Ulven; smulven@medisin.uio.no,

Kirsten B. Holven; k.b.holven@medisin.uio.no

Corresponding author; Kirsten B. Holven, Department of Nutrition, Institute of Basic Medical Sciences, Faculty of Medicine, P.O. Box 1046, Blindern, 0317 University of Oslo, Norway.

A list of authors' last name, as they should appear for PubMed indexing: Gjevestad, Hamarsland, Raastad, Ottestad, Christensen, Eckardt, Drevon, Biong, Ulven, Holven

Number of figures to print: 6

Number of tables to print: 7

Abstract

Background: High-intensity exercise induces many metabolic responses. In is unknown whether the response in peripheral blood mononuclear cells (PBMCs) reflects the response in skeletal muscle, and whether mRNA expression after exercise can be modulated by nutritional intake.

Objective: The aims were to; i) investigate the effect of dairy proteins on acute responses to exercise in skeletal muscle and PBMCs measuring gene expression and ii) compare this response in young and older subjects.

Methods: We performed two separate studies in young (20-40 yrs) and older subjects (≥ 70 yrs). Subjects were randomly allocated to a milk group or a whey group. Supplements were provided immediately after a standardized exercise session. We measured mRNA expression of selected genes after a standardized breakfast and 60/120 min after finishing the exercise, using RT-qPCR.

Results: We observed no significant differences in mRNA expression between the milk and the whey group, thus, we merged both groups for further analysis. The mRNA expression of *IL6, TNF* and *CCL2* in skeletal muscle increased significantly after exercise compared with smaller, or no increase, in mRNA expression in PBMCs in all participants. The mRNA expression of *IL1RN, IL8* and *IL10* increased significantly in skeletal muscle and PBMCs. Some mRNA transcripts were differently regulated in older compared to younger participants in PBMCs.

Conclusion: An acute bout of heavy-load strength exercise, followed by protein supplementation, caused overlapping, but also unique, responses in skeletal muscle and PBMCs, suggesting tissue specific functions in response to exercise. However, no different effects of the different protein supplements were observed. Altered mRNA expressions in PBMCs of older participants may affect regenerative mechanisms.

Key words: peripheral blood mononuclear cells, skeletal muscle, mRNA, resistance exercise, muscle regeneration, cytokines.

Background

High-intensity physical exercise induces several metabolic responses and represents a major challenge to whole-body homeostasis. Numerous adaptations take place to meet this challenge, both locally (including changes in mRNA expression and protein levels) and systemically (including hormonal signaling and organ crosstalk) [1-3]. Ultimately, these events will promote altered expression of key proteins in the skeletal muscle [4-6], as well as the immune system [5, 7, 8]. To study alternations in gene expression levels of the immune system in response to short- and long-term nutritional interventions, peripheral blood mononuclear cells (PBMCs) have been used as a surrogate model [9, 10]. Whether PBMCs are a good model system for studying gene expression levels in skeletal muscle in response to exercise is less known.

A growing number of studies show that mRNA expression in the recovery phase from exercise can be modulated by nutritional intake [11-16]. Intake of sufficient energy and protein, in combination with regular exercise, may promote muscle protein synthesis [17-20]. Dairy proteins have been hypothesized to modulate inflammation by having anti-inflammatory properties [21-23]. However, we know little about how different dairy proteins affect mediators of the immune system after an acute bout of exercise.

Aging is associated with a range of cellular and biochemical changes, including increased inflammation, altered cell migration and cell signaling [24], and may be an important factor determining the molecular signature of an acute bout of exercise. Previous studies suggest an attenuated expression of markers released after exercise in old compared to young subjects, in skeletal muscle [25-32], in serum [33] as well as in cells of the immune system [34].

The aims of the present study were to; i) investigate the effect of dairy proteins on acute responses to exercise in skeletal muscle and PBMCs measuring mRNA expression of selected genes and ii) compare this response in young and older subjects.

Methods

Study populations and experimental design

We performed two separate acute exercise studies, where we supplemented young (20-45 yrs) and older (≥ 70 yrs) adults with dairy products based on regular milk or whey protein. Both studies were conducted at the Norwegian School of Sports Sciences. The first study (study 1) was conducted during the fall of 2013 and the second study (study 2) during the fall of 2014 and the spring of 2015. Written informed consent was obtained from all participants.

In study 1, 24 resistance trained young (mean age; 25.0 ± 3.5 yrs) and 17 recreationally active older (mean age; 74.2 ± 3.8) healthy men and women were randomly allocated into a milk group or a whey group. Further, subjects in the whey group were randomized to receive whey protein concentrate (WPC80) or native whey on the first test day in a crossover design (Figure 1a). Information about the training habits the last six months before inclusion were obtained. In study 2, 25 young (mean age; 28.9 ± 5.8 yrs) and 24 older (mean age; 73.7 ± 3.4 yrs) healthy, untrained men and women, were randomized into a milk group or a native whey group (Figure 1b). In both studies, the appropriate test drink was consumed immediately after performing a standardized exercise session. Participants reported to be non-smokers with no cardiovascular diseases or diabetes. In study 1, three older subjects had prescribed medication for high blood pressure and two took statins. In study 2, one older subject had prescribed anticoagulants and six took statins. One older participant and two young subjects did not complete the study and were excluded from further analysis in study 1, whereas one young participant did not complete the study and was excluded from further analysis in study 2.

On the morning of each test day, subjects reported to the laboratory in a fasted state. Upon arrival, they were served a standardized breakfast consisting of oatmeal, water, rapeseed oil and sugar (50 energy percent (E%) from carbohydrate, 8 E% from protein and 42 E% from fat). All subjects finished the breakfast within 20 min. One day before the exercise, and until the last performance test was completed the following day, all participants followed a standardized diet.

Protein supplements

The supplements were based on regular milk or whey protein (WPC80 or native whey proteins). The test products were isocaloric and contained 20 g of protein (27 E%), 39 g carbohydrates (52 E%) and 7 g fat (21 E%), providing approximately 300 kcal per serving.

Thus, the main difference between test products were the amino acid composition, as illustrated in Table 1. Further, the production method for WPC80 differed from that of native whey as native whey was produced at low temperatures (below 60°C). In both studies, the supplements were provided in identical packages to ensure blinding of both the providers and the participants, although the products were labeled with color codes to ensure that the participants received the correct products. The color-coding was provided by the producer and was not revealed until the interventions and statistical analyzes were completed. All products had the same flavor, color and appearance.

Exercise protocols

In study 1, the young participants had experience with strength training prior to inclusion, whereas the older subjects had been recreationally active. To become accustomed with the exercise session, young participants performed the exercise session twice prior to the test day, whereas older subjects performed the exercise session until they were familiar with the exercise (average 4.4 times, maximum of 6 times). These exercise sessions were also used to determine the workload needed for each participant. On the test day, the exercise session lasted for 30 min and included 4x8 repetition maximum (RM) of leg press and knee extension, with a new set starting every third min. Baseline was defined as 2.5 h after the standardized breakfast was served. The exercise session started approximately 30 min after baseline, and was immediately followed by intake of a test drink (3.5 h after breakfast was served). Subjects had to finish the test drink within five min. Blood samples and skeletal muscle biopsies were collected at baseline and 1 h after completing the exercise (Figure 2).

Participants in study 2 were untrained, but they were made familiar to the exercise session and the 10 RM training loads were determined. On the test day, the exercise session lasted for 45 min and included 3x10 RM of hammer squat, leg press, knee extension, bench press (chest press in the older subjects), seated rowing and 1x10 RM and 2x10 RM in close grip pull-down and shoulder press, respectively. A new set started every third min. In this study, baseline was defined as 1 h after breakfast was served. The exercise session started approximately 30 min after baseline, and was immediately followed by the intake of a test drink (2.15 h after breakfast was served), which had to be finished within five min. Blood samples and skeletal muscle biopsies were collected at baseline and 2 h after completing the exercise (Figure 2).

Sampling and sample preparation

Venous blood samples were collected in BD Vacutainer® CPT™ cell preparation tubes with sodium heparin (Becton Dickinson, NJ, USA) and in BD Vacutainer® SST™II Advanced tubes for serum (Becton Dickinson, NJ, USA). Within 2 h of blood collection, PBMCs were collected by density gradient centrifugation (1636 *g*) for 25 min at room temperature. The cells were washed twice (300 *g*, 10 min) in PBS without $CaCl_2$ and $MgCl_2$. After the last washing step, excess PBS was discarded. The pellet was dissolved in the remaining liquid and transferred to an Eppendorf tube, centrifuged (13000 *g*, 3 min, 4°C) and frozen at -80°C until further analysis. Serum samples were left on the bench top for at least 30 min to ensure complete coagulation, centrifuged (1550 *g*, 15 min at room temperature) and frozen at -80°C until further analysis.

Muscle biopsies from *m. vastus lateralis* were collected at the same time points as the blood samples with a modified Bergstrom technique [35]. The biopsies were immediately cleaned from blood and connective tissue in physiological salt water at 4˚C, immersed into RNAlater® solution (Ambion, Texas, USA) and stored overnight at 4˚C. The following day, the biopsies were transferred and stored at – 80 ˚C until further analysis. Biopsies were taken from the left leg, and the same incision was used for both biopsies, but the needle was placed in an angle so that the two samples sites were separated by at least five centimeters. The second sample was always collected proximal to the first sample.

Isolation of mRNA

mRNA was isolated from thawed PBMCs using Qiagen RNease Mini Kit in accordance with the protocol provided (QIAGEN GmbH, Germany). In brief, PBMC pellets were lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer. Ethanol was added to provide appropriate binding conditions before the samples were applied to an RNeasy Mini spin column. Contaminants were washed out using buffers in the kit. A one-column DNase digest (QIAGEN GmbH, Germany) was used to remove potential DNA contaminants. High-quality RNA was eluted in 30 µL of RNase free water and frozen at -80 °C until further analysis. In study 2 this protocol was conducted using the QiaCube (QIAGEN GmbH, Germany) in accordance with the protocol RNeasy Mini Kit with Qiashredder columns and DNase digest. High-quality RNA was eluted in 30 µL RNAse free water and frozen at -80 ˚C until further analysis.

Without thawing, muscle biopsies were ruptured using a mortar and pestle (study 1) or a CryoGrinder (study 2), followed by homogenization in Qiazol (QIAGEN GmbH, Germany).

Chloroform (1:5, v:v for chloroform:Qiazol) was added and samples centrifuged (12000 *g*, 15 min, 4°C). The upper phase, with mRNA, was transferred to a fresh tube before adding ethanol. The samples were applied to a miRNeasy column using the protocol provided by QIAGEN GmbH (Germany). The protocol was performed manually in study 1, whereas the QiaCube from QIAGEN GmbH (Germany) was used in study 2. 30 µL high-quality RNA was eluted in RNase free water and frozen at -80 °C until further analysis.

RNA quantity was measured using NanoDrop-1000 (NanoDrop Technologies, Inc., Delaware, USA), and RNA quality was checked with Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., California, USA). All samples included in further analyses had a RIN-value above 6. One young participant from study 1 and two subjects (one young and one older) from study 2 were excluded from further analysis, due to missing PBMC samples. Five participants (one young and four older) and 19 participants (15 young and six older) from study 1 and 2, respectively, were excluded from further analysis due to low RNA quality isolated from the muscle samples.

Synthesis of cDNA

Complementary DNA (500 ng) was made using a RNA to cDNA kit from Applied Biosystems (Applied Biosystems, UK) in accordance with the manufacturer`s protocol. Samples were stored at -20 °C for further analysis.

RNA analysis by RT-qPCR

We monitored the mRNA expression levels of 24 mRNA transcripts, using TaqMan Low-Density array (TLDA) cards from Applied Biosystems (UK). Eighteen of the transcripts were analyzed in both studies (Additional file 1; overview of mRNA transcripts). mRNA transcripts were selected based on a thorough literature search investigating the effect of acute exercise on gene expression levels in PBMCs [36] as well as skeletal muscle [37]. Moreover, the selection was based on studies where effects of dairy products on markers of inflammation were investigated [38]. TLDA cards were used on a 7900 HT Applied Biosystems RT-qPCR machine (Applied Biosystems, UK). The Ct-values were analyzed using SDS 2.4 (Applied Biosystems, UK), and further transferred to ExpressionSuite Sofware v1.0.3 (Applied Biosystems, UK). We normalized the Ct-values to TATA box binding protein (TBP) mRNA transcripts. Fold changes in mRNA transcripts from baseline to after the exercise session, were calculated by dividing $2^{-\Delta Ctpost\ exercise}$ with $2^{-\Delta Ctbaseline}$, using the $2^{-\Delta\Delta Ct}$-method [39].

Cytokine measurements

The serum level of IL6 was determined using a high sensitivity Quantikine HS ELISA Kit (R&D Systems Inc., Minneapolis, USA), according to the protocol. All samples were measured in duplicates.

Statistics

Power calculations were only made for the primary outcome of the study, but number of participants included in the mRNA expression analysis reported here, is in line with the number included in similar studies exploring potential changes of exercise on mRNA expression levels [40-44].

First, we examined changes in mRNA expression and serum levels from the acute bout of exercise between drinks, as well as between young and older subjects, in both studies separately. Since we found no differences in gene expression and serum levels between WPC80 and native whey in study 1, we decided to combine the results from the whey groups when comparing the whey group with the milk group. After inspecting the results of study 1 and study 2 individually, we also found these to be similar, thus, we decided to combine the results from study 1 and study 2 into one data set.

All data were checked for normality. For non-parametric data, we used Wilcoxon signed rank test for repeated, paired measurements, and the Mann-Whitney test for independent measurements. Fold changes (relative quantification) were calculated using the ratio of $2^{-\Delta Ct}$ $^{\text{after exercise}}$ to $2^{-\Delta Ct}$ $^{\text{baseline}}$ [39]. For parametric data, differences between study groups at baseline were performed by the independent sample t-test.

Due to the explorative study design, we performed no correction for multiple testing, and we considered a p-value of < 0.05 statistically significant. We used SPSS statistical software (SPSS), version 22, from Microsoft (SPSS, Inc., Chicago, USA) for statistical calculations and GraphPad Prism 5 (GraphPad Software, Inc., California, USA) for creating figures.

Results

Initial statistical analysis showed no differences in mRNA transcripts between the WPC80 and the native whey groups in study 1, in neither young nor older participants. We therefore decided to combine the whey groups in further analysis. Furthermore, we combined the data sets from study 1 and study 2 because the design became similar in these two studies when merging the whey groups in study 1. Moreover, the mRNA responses were similar in the two studies. Thus, in this paper, we report the mRNA expression levels of selected genes in skeletal muscle and PBMCs from two acute strength exercise studies combined where exercise was combined with supplementation with milk or whey protein.

Baseline characteristics in young and older subjects, independently of the supplement provided, showed that fat percent and leg lean mass were significantly different between young and older participants (Table 2).

Adherence to the exercise protocols

The average training volume in study 1 was 9050 ± 2197 kg and 5634 ± 2307 kg for the young and older subjects, respectively. The relative work-load was the same for both groups (8 RM). All exercise sessions were performed as planned, but some subjects, in both groups, needed assistance with the last repetition and some seconds extended break before the last set.

The average training volume in study 2 was 11852 ± 3083 kg and 5982 ± 2458 kg for the young and older subjects, respectively. The relative work-load was similar for both groups (10 RM). In study 2, three young and two older subjects did not go through with the shoulder press exercise due to shoulder pain. Except for this adjustment, all exercise sessions were performed as planned.

Effects of exercise and protein supplementation on mRNA expression

We observed that the mRNA expression levels of *IL6, TNF* and *CCL2* in skeletal muscle increased significantly after acute exercise compared with smaller or no increase in mRNA expression levels in PBMCs (Figure 3). The mRNA expression levels of *IL1RN*, *IL8* and IL10 increased significantly in both skeletal muscle and PBMCs after exercise, but the expression levels of *IL1RN* and *IL8* were higher expressed in skeletal muscle after exercise than in PBMCs (Figure 4, panel A-D and Figure 5, panels C-D). The expression level of *IL10* was similar in skeletal muscle and PBMCs. The mRNA expression level of *IL1β* was significantly

enhanced by exercise in skeletal muscle, whereas a significant increase of *IL1β* after exercise in PBMCs was observed in younger participants only (Figure 5, panels A-B).

Older participants showed an attenuated response in the mRNA expression level of *IL10* in PBMCs, whereas the mRNA expression levels of *IL8* and *IL1β* were higher in older compared to young participants. The mRNA expression level of *CCL3* decreased in both young and older participants, but the decrease was less pronounced in older participants. No significant changes in mRNA expression levels were observed between young and older participants in skeletal muscle (Figure 4, panels C-F, Figure 5, panels A-D). Baseline mRNA expression levels of *IL8*, *IL10* and *CCL3* in PBMCs were higher in older compared to younger participants. The mRNA expression levels in skeletal muscle and PBMCs of all genes measured, in both young and older subjects, are shown in the Additional file 2.

Effect of exercise and protein supplementation on serum IL6

We observed a significant increase in the circulating level of IL6 after exercise in both young and older subjects (Figure 6), with no significant difference between the two groups. Serum concentration of IL6 was significantly higher in older than in younger subjects at baseline ($p<0.001$).

Discussion

We observed that acute exercise modulated changes in mRNA expression levels of several genes known to be involved in repair, regeneration and adaptive processes of exercise, in skeletal muscle as well as PBMCs. Some of the changes observed were regulated similarly in skeletal muscle and PBMCs, whereas other mRNA transcripts showed a unique pattern. Furthermore, we observed an attenuated response in the PBMC mRNA expression level of *IL10* in older compared to younger participants, whereas the PBMC mRNA expression levels of *IL8* and *IL1β* increased in older compared to younger participants after exercise. In contrast, the intake of different types of dairy protein had no significant impact on the mRNA response neither in skeletal muscle nor in PBMCs.

When comparing mRNA expression levels in skeletal muscle and PBMCs, we observed three different expression patterns; i) mRNA transcripts increased significantly in skeletal muscle only ii) mRNA transcripts increased significantly in both skeletal muscle and PBMCs, but the magnitude of the increase was higher in skeletal muscle than in PBMCs and iii) mRNA transcripts were similarly expressed in both skeletal muscle and PBMC. These results demonstrate that skeletal muscle and PBMCs have overlapping, as well as unique mRNA responses to acute exercise, suggesting tissue specific functions in response to acute exercise. IL6 has for example consistently been shown to increase in skeletal muscle and in serum after acute exercise [26, 45-49], whereas data from PBMC mRNA expression analysis showed a modest, or no increase of IL6 after acute exercise [36]. A temporary increase in IL6 after exercise may affect satellite cells and promote myogenic lineage progression [50-53]. TNF and IL1β may have a role in promoting myoblast proliferation [54], and in inhibiting myoblast differentiation [55], potentially being important contributors to skeletal muscle adaptions. In PBMCs, the function of TNF and IL1β are primarily pro-inflammatory, playing a less important role after acute exercise. In addition, we observed a significant increase in mRNA expression levels of *IL10* and *IL1RN* after exercise in both skeletal muscle and PBMCs. We speculate that the increased mRNA expression levels of these cytokines may induce a regenerative response in patrolling PBMCs, possibly as an attempt to restore homeostasis [53, 56, 57]. The increase of these cytokines in PBMCs may be a result of IL6 released from skeletal muscle after acute exercise [57-60]. In the present study, the mRNA expression of *CCL2* was strongly increased in skeletal muscle, whereas no change was observed in PBMCs. Little is known about the function and physiological relevance of CCL2

after strength exercise in humans, but studies on contracting C2C12 myotubes show that CCL2 is released in a NF-κB-dependent manner to induce monocyte chemoattraction [61].

Few studies have been performed investigating the response to acute exercise in skeletal muscle and PBMCs simultaneously, but Liburt and colleagues observed that mRNA expression levels of *IL6* and *TNF* increased in skeletal muscle after acute exercise in horses, with no increase in PBMCs. They also found that the expression of *IL1* was similar in skeletal muscle and PBMCs [62]. Zeibig and colleagues found a significant correlation of mRNA transcripts of mitochondrial carnitine acyltransferases between skeletal muscle and human blood cells after 6 months of endurance exercise in young men [63], whereas Rudkowska and colleagues reported that 88 % of the mRNA transcripts in skeletal muscle and PBMCs overlapped after eight weeks of supplementation with n-3 polyunsaturated fatty acids using a transcriptome approach [9].

Further, we observed both a reduced and an increased response to acute exercise in older compared to younger participants in PBMCs, with no differences in skeletal muscle. Few, if any, have investigated possible differences in mRNA expression levels of PBMCs after acute exercise between young and older subjects. An attenuated cytokine response to acute exercise in older subjects has been observed in serum [33], with conflicting results in skeletal muscle [26, 28, 30, 64]. Knowing that immune cells may be an important part of adaptive processes to exercise [30], an altered response of cytokines and chemokines in PBMCs of older subjects may ultimately impair regeneration. In the present study, the relative workload was the same in young and older participants, but the training volume differed between the groups. Younger participants were stronger and able to lift a higher load than the older participants. This may have contributed to a higher systemic stress in younger than in older participants, possibly being part of the explanation for the altered response observed in PBMCs between younger and older participants.

No differences in mRNA expression levels of investigated markers were observed 60-120 min after the heavy-load strength exercise, depending on the protein source (whey or milk). These results were supported by Nieman and colleagues who reported no differences in mRNA expression levels of markers, such as *IL6, IL1β, IL8* and *IL10*, after an intense resistance exercise session, combined with the consumption of supplements consisting of carbohydrate (50%), protein (16 %) and fat (34 %) [12]. In contrast, other studies have indicated that whey proteins may have anti-inflammatory properties, by limiting the activation of NF-κB [21, 56].

In stimulated PBMCs, cultured in the presence of different glutamine concentrations, glutamine may enhance the production of T lymphocyte-derived cytokines, such as IL10 [15]. Similarly, glucose ingestion may attenuate IL6 release from contracting skeletal muscle after 120 min of cycling [16]. The time course of mRNA expression may differ in endurance and strength exercise, possibly explaining the different results.

There are some limitations to the present study. We report one post-exercise time point only, which limits our ability to identify potential differences in the time course of transcriptional regulation that may result from training or supplementation. This sampling point may also have been too early to detect possible differences in mRNA expression levels of the protein source provided. Another limitation of the study were that even though we investigating the effect of a relative work load, because the young subjects were stronger and lifted approximately twice the volume of the old participants it is not unreasonable to assume that the relative systemic stress (e.g. circulatory system etc.) was higher in the young and we therefore cannot exclude the possibility that this may have contributed to different responses in PBMC. Major strengths to the present study are the randomized controlled design, with participants receiving a standardized diet prior to, and on the test day, and the standardized exercise sessions that were performed under close supervision. Blood samples and muscle biopsies were collected simultaneously allowing comparison of the responses in two tissues.

Conclusions

We report changes in mRNA expression levels of selected genes, in skeletal muscle and PBMCs, after two acute bouts of strength exercise, followed by the intake of different protein supplements, in young and old participants. There were both overlapping and unique responses in mRNA transcripts of skeletal muscle and PBMCs in response to high-load exercise, suggesting tissue specific functions in response to acute exercise. Furthermore, we observed that there were some differences in mRNA response to exercise in young and old subjects in PBMCs, possibly affecting regenerative mechanisms. Finally, our results show that different dairy protein supplements did not differentially alter mRNA transcripts after exercise.

Abbreviations list

CCL, chemokine (C-C motif) ligand; E%, energy percent; IL, interleukin; IL1RN, interleukin 1 receptor antagonist; IMVC, isometric maximum voluntary contraction; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NR4A2; nuclear receptor subfamily 4, group A, member 2; PBMCs, peripheral blood mononuclear cells; PPARGC1A; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, RM, repetition maximum; RT-qPCR, real-time quantitative polymerase chain reaction; TBP, TATA box binding protein; TLDA, TaqMan Low-Density array; TLR2, toll-like receptor 2; TNF, tumor necrosis factor alpha; VO2max, maximal oxygen uptake; WPC80, whey protein concentrate

Declarations

Ethic approval

Both studies were approved by the National Committee for Research Ethics, Oslo, Norway (2014/834), and performed according to the Declaration of Helsinki (last amended 2008).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The test products were provided by TINE SA, Oslo, Norway, where G.O.G. and A.S.B. are researchers employed, G.O.G. as an industrial PhD-student. They have no financial interest to declare. I.O., T.R., H.H., J.J.C., K.E. C.A.D., K.B.H. and S.M.U. report no conflict of interest.

K.B.H. has received research grant from TINE SA, Mills DA, Olympic Seafood, Amgen, Sanofi and Pronova. S.M.U. has received research grant from TINE SA, Mills DA and Olympic Seafood. T.R. has received grants from TINE SA. KE is supported by the Deutsche Forschungsgemeinschaft (German Research Foundation; EC 440/2-1).

Funding

The work was supported by The Research Council of Norway (project number 225258/E40), Throne Holst Foundation for Nutrition Research (University of Oslo), The Norwegian School of Sports Sciences (NIH) and TINE SA. The University of Oslo, NIH and TINE SA designed the study. The University of Oslo and NIH were responsible for data collection, analysis, interpretations of the data and writing the manuscript.

Authors' contributions

Conception or design of the study; T.R., H.H., A.S.B., S.M.U. and K.B.H. Acquisition, analysis or interpretation of the work; all authors. Drafting or critically revising the manuscript; all authors. Read and approved the final manuscript; all authors.

Acknowledgements

We want to acknowledge all the participants volunteering to participate in these studies, and Torgeir Holen for sharing techniques and treasured experience about RNA isolation from skeletal muscle biopsies.

Consent for publication

Not applicable.

Open Access

Additional files

Additional file 1; file format; docx, Title; mRNA transcripts analyzed in both studies. Description; Overview of mRNA transcripts analyzed in the present study.

Additional file 2; file format; docx, Title; mRNA expression levels in skeletal muscle and PBMCs of young and older subjects. Description; Baseline and after exercise (post exercise) values, expressed as $2^{-\Delta Ct.}$.

Reference list

1. Egan, B., J.A. Hawley, and J.R. Zierath, *SnapShot: Exercise Metabolism.* Cell Metab, 2016. **24**(2): p. 342-342.e1.
2. Pillon, N.J., et al., *Cross-talk between skeletal muscle and immune cells: muscle-derived mediators and metabolic implications.* Am J Physiol Endocrinol Metab, 2013. **304**(5): p. E453-65.
3. Gorgens, S.W., et al., *Exercise and Regulation of Adipokine and Myokine Production.* Prog Mol Biol Transl Sci, 2015. **135**: p. 313-36.
4. Chorell, E., et al., *Predictive metabolomics evaluation of nutrition-modulated metabolic stress responses in human blood serum during the early recovery phase of strenuous physical exercise.* J Proteome Res, 2009. **8**(6): p. 2966-77.
5. Calle, M.C. and M.L. Fernandez, *Effects of resistance training on the inflammatory response.* Nutr Res Pract, 2010. **4**(4): p. 259-69.
6. Norheim, F., et al., *Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training.* Am J Physiol Endocrinol Metab, 2011. **301**(5): p. E1013-21.
7. Pedersen, B.K., *Muscle as a secretory organ.* Compr Physiol, 2013. **3**(3): p. 1337-62.
8. Gleeson, M., *Immune function in sport and exercise.* J Appl Physiol (1985), 2007. **103**(2): p. 693-9.
9. Rudkowska, I., et al., *Validation of the use of peripheral blood mononuclear cells as surrogate model for skeletal muscle tissue in nutrigenomic studies.* Omics, 2011. **15**(1-2): p. 1-7.
10. de Mello, V.D., et al., *Gene expression of peripheral blood mononuclear cells as a tool in dietary intervention studies: What do we know so far?* Mol Nutr Food Res, 2012. **56**(7): p. 1160-72.
11. Starkie, R.L., et al., *Carbohydrate ingestion attenuates the increase in plasma interleukin-6, but not skeletal muscle interleukin-6 mRNA, during exercise in humans.* J Physiol, 2001. **533**(Pt 2): p. 585-91.
12. Nieman, D.C., et al., *Influence of carbohydrate ingestion on immune changes after 2 h of intensive resistance training.* J Appl Physiol (1985), 2004. **96**(4): p. 1292-8.
13. Nieman, D.C., et al., *Carbohydrate ingestion influences skeletal muscle cytokine mRNA and plasma cytokine levels after a 3-h run.* J Appl Physiol (1985), 2003. **94**(5): p. 1917-25.
14. Hawley, J.A., K.D. Tipton, and M.L. Millard-Stafford, *Promoting training adaptations through nutritional interventions.* J Sports Sci, 2006. **24**(7): p. 709-21.
15. Yaqoob, P. and P.C. Calder, *Cytokine production by human peripheral blood mononuclear cells: differential senstivity to glutamine availability.* Cytokine, 1998. **10**(10): p. 790-4.
16. Febbraio, M.A., et al., *Glucose ingestion attenuates interleukin-6 release from contracting skeletal muscle in humans.* J Physiol, 2003. **549**(Pt 2): p. 607-12.
17. Cermak, N.M., et al., *Protein supplementation augments the adaptive response of skeletal muscle to resistance-type exercise training: a meta-analysis.* Am J Clin Nutr, 2012. **96**(6): p. 1454-64.
18. Nicastro, H., et al., *Does Branched-Chain Amino Acids Supplementation Modulate Skeletal Muscle Remodeling through Inflammation Modulation? Possible Mechanisms of Action.* J Nutr Metab, 2012. **2012**: p. 136937.
19. Morley, J.E., et al., *Nutritional recommendations for the management of sarcopenia.* J Am Med Dir Assoc, 2010. **11**(6): p. 391-6.
20. Paddon-Jones, D. and B.B. Rasmussen, *Dietary protein recommendations and the prevention of sarcopenia.* Curr Opin Clin Nutr Metab Care, 2009. **12**(1): p. 86-90.
21. Madureira, A.R., et al., *Invited review: physiological properties of bioactive peptides obtained from whey proteins.* J Dairy Sci, 2010. **93**(2): p. 437-55.

22. Draganidis, D., et al., *Inflammaging and Skeletal Muscle: Can Protein Intake Make a Difference?* J Nutr, 2016.
23. McGregor, R.A. and S.D. Poppitt, *Milk protein for improved metabolic health: a review of the evidence.* Nutr Metab (Lond), 2013. **10**(1): p. 46.
24. Chung, H.Y., et al., *Molecular inflammation: underpinnings of aging and age-related diseases.* Ageing Res Rev, 2009. **8**(1): p. 18-30.
25. Dennis, R.A., et al., *Aging alters gene expression of growth and remodeling factors in human skeletal muscle both at rest and in response to acute resistance exercise.* Physiol Genomics, 2008. **32**(3): p. 393-400.
26. Hamada, K., et al., *Senescence of human skeletal muscle impairs the local inflammatory cytokine response to acute eccentric exercise.* FASEB J, 2005. **19**(2): p. 264-6.
27. Jozsi, A.C., et al., *Molecular characteristics of aged muscle reflect an altered ability to respond to exercise.* Int J Sport Nutr Exerc Metab, 2001. **11 Suppl**: p. S9-15.
28. Jozsi, A.C., et al., *Aged human muscle demonstrates an altered gene expression profile consistent with an impaired response to exercise.* Mech Ageing Dev, 2000. **120**(1-3): p. 45-56.
29. Merritt, E.K., et al., *Heightened muscle inflammation susceptibility may impair regenerative capacity in aging humans.* J Appl Physiol (1985), 2013. **115**(6): p. 937-48.
30. Przybyla, B., et al., *Aging alters macrophage properties in human skeletal muscle both at rest and in response to acute resistance exercise.* Exp Gerontol, 2006. **41**(3): p. 320-7.
31. Raue, U., et al., *Proteolytic gene expression differs at rest and after resistance exercise between young and old women.* J Gerontol A Biol Sci Med Sci, 2007. **62**(12): p. 1407-12.
32. Thalacker-Mercer, A.E., et al., *Differential genomic responses in old vs. young humans despite similar levels of modest muscle damage after resistance loading.* Physiol Genomics, 2010. **40**(3): p. 141-9.
33. Toft, A.D., et al., *Cytokine response to eccentric exercise in young and elderly humans.* Am J Physiol Cell Physiol, 2002. **283**(1): p. C289-95.
34. Gano, L.B., et al., *Increased proinflammatory and oxidant gene expression in circulating mononuclear cells in older adults: amelioration by habitual exercise.* Physiol Genomics, 2011. **43**(14): p. 895-902.
35. Paulsen, G., et al., *Vitamin C and E supplementation alters protein signalling after a strength training session, but not muscle growth during 10 weeks of training.* J Physiol, 2014. **592**(24): p. 5391-408.
36. Gjevestad, G.O., K.B. Holven, and S.M. Ulven, *Effects of Exercise on Gene Expression of Inflammatory Markers in Human Peripheral Blood Cells: A Systematic Review.* Curr Cardiovasc Risk Rep, 2015. **9**(7): p. 34.
37. Paulsen, G., et al., *Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise?* Exerc Immunol Rev, 2012. **18**: p. 42-97.
38. Labonte, M.E., et al., *Impact of dairy products on biomarkers of inflammation: a systematic review of randomized controlled nutritional intervention studies in overweight and obese adults.* Am J Clin Nutr, 2013. **97**(4): p. 706-17.
39. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
40. Ulven, S.M., et al., *An acute bout of exercise modulate the inflammatory response in peripheral blood mononuclear cells in healthy young men.* Arch Physiol Biochem, 2015: p. 1-9.
41. Storey, A.G., et al., *Stress responses to short-term intensified and reduced training in competitive weightlifters.* Scand J Med Sci Sports, 2015.
42. Ostrowski, K., et al., *Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running.* J Physiol, 1998. **508 (Pt 3)**: p. 949-53.
43. Louis, E., et al., *Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle.* J Appl Physiol (1985), 2007. **103**(5): p. 1744-51.

44. Della Gatta, P.A., D. Cameron-Smith, and J.M. Peake, *Acute resistance exercise increases the expression of chemotactic factors within skeletal muscle.* Eur J Appl Physiol, 2014. **114**(10): p. 2157-67.
45. Pedersen, B.K. and M.A. Febbraio, *Muscles, exercise and obesity: skeletal muscle as a secretory organ.* Nat Rev Endocrinol, 2012. **8**(8): p. 457-65.
46. Brown, W.M., et al., *A Systematic Review of the Acute Effects of Exercise on Immune and Inflammatory Indices in Untrained Adults.* Sports Med Open, 2015. **1**(1): p. 35.
47. Buford, T.W., et al., *Effects of eccentric treadmill exercise on inflammatory gene expression in human skeletal muscle.* Appl Physiol Nutr Metab, 2009. **34**(4): p. 745-53.
48. Steensberg, A., et al., *IL-6 and TNF-alpha expression in, and release from, contracting human skeletal muscle.* Am J Physiol Endocrinol Metab, 2002. **283**(6): p. E1272-8.
49. Langleite, T.M., et al., *Insulin sensitivity, body composition and adipose depots following 12 w combined endurance and strength training in dysglycemic and normoglycemic sedentary men.* Arch Physiol Biochem, 2016: p. 1-13.
50. Serrano, A.L., et al., *Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy.* Cell Metab, 2008. **7**(1): p. 33-44.
51. Toth, K.G., et al., *IL-6 induced STAT3 signalling is associated with the proliferation of human muscle satellite cells following acute muscle damage.* PLoS One, 2011. **6**(3): p. e17392.
52. Tierney, M.T., et al., *STAT3 signaling controls satellite cell expansion and skeletal muscle repair.* Nat Med, 2014. **20**(10): p. 1182-6.
53. Chazaud, B., *Inflammation during skeletal muscle regeneration and tissue remodeling-application to exercise-induced muscle damage management.* Immunol Cell Biol, 2015.
54. Otis, J.S., et al., *Pro-inflammatory mediation of myoblast proliferation.* PLoS One, 2014. **9**(3): p. e92363.
55. Langen, R.C., et al., *Inflammatory cytokines inhibit myogenic differentiation through activation of nuclear factor-kappaB.* Faseb j, 2001. **15**(7): p. 1169-80.
56. Cruzat, V.F., M. Krause, and P. Newsholme, *Amino acid supplementation and impact on immune function in the context of exercise.* J Int Soc Sports Nutr, 2014. **11**(1): p. 61.
57. Lancaster, G.I. and M.A. Febbraio, *The immunomodulating role of exercise in metabolic disease.* Trends Immunol, 2014. **35**(6): p. 262-9.
58. Suzuki, K., et al., *Systemic inflammatory response to exhaustive exercise. Cytokine kinetics.* Exerc Immunol Rev, 2002. **8**: p. 6-48.
59. Petersen, A.M. and B.K. Pedersen, *The anti-inflammatory effect of exercise.* J Appl Physiol (1985), 2005. **98**(4): p. 1154-62.
60. Mathur, N. and B.K. Pedersen, *Exercise as a mean to control low-grade systemic inflammation.* Mediators Inflamm, 2008. **2008**: p. 109502.
61. Miyatake, S., et al., *Contracting C2C12 myotubes release CCL2 in an NF-kappaB-dependent manner to induce monocyte chemoattraction.* Am J Physiol Endocrinol Metab, 2016. **310**(2): p. E160-70.
62. Liburt, N.R., et al., *Exercise-induced increases in inflammatory cytokines in muscle and blood of horses.* Equine Vet J Suppl, 2010(38): p. 280-8.
63. Zeibig, J., et al., *Do blood cells mimic gene expression profile alterations known to occur in muscular adaptation to endurance training?* Eur J Appl Physiol, 2005. **95**(1): p. 96-104.
64. Ivey, F.M., et al., *Effects of age, gender, and myostatin genotype on the hypertrophic response to heavy resistance strength training.* J Gerontol A Biol Sci Med Sci, 2000. **55**(11): p. M641-8.

Figure legends

Figure 1a. In study 1, participants were randomized into into one of two group, receiving either milk og whey supplements. Participants in the whey group were testing two different whey products in a randomized order. All groups performed the exercise. There was minimum one week between test days in the crossover part of the study.

Figure 1b. In study 2, participants were randomized into one of two groups, receiving either milk og native whey supplements in combination with exercise.

Figure 2. Timeline of study design and times of sampling in study 1 and study 2. A standardized breakfast was served upon arrival, 2.5 h (study 1) or 1 h (study 2) before the baseline samples were drawn. 30 min after baseline, a 30 (study 1) or 45 (study 2) min exercise session was performed directly followed by intake of a protein drink. Post exercise samples were drawn 1 (study 1) or 2 (study 2) h after finishing the drink.

Figure 3. mRNA expression levels of *IL6* [A, B], *TNF* [C, D], and *CCL2* [E, F] after an acute bout of strength exercise, expressed as fold changes, in young and older subjects in skeletal muscle and PBMC. Skeletal muscle (young subjects); n = 25 [C], n = 28 [A] and n = 30 [E]. Skeletal muscle (older subjects); n = 27 [C], n = 28 [E], and n = 29 [A]. PBMC (young subjects); n = 40 [D, F] and n=39 [B]. PBMC (older subjects) ; n = 31 [B, D, F]. Data are shown as median and interquartile ranges.

Figure 4. mRNA expression levels of *IL1RN* [A, B], *IL8* [C, D] and *CCL3* [E, F] after an acute bout of strength exercise, expressed as fold changes, in young and older subjects in skeletal muscle and PBMC. Skeletal muscle (young people); n = 18 [E], n = 20 [C] and n = 22 [A]. Skeletal muscle (older subjects); n = 22 [E], n = 23 [C], n = 25 [A]. PBMC (young subjects) ; n = 38 [D], n = 39 [F] and n = 40 [B]. PBMC (older subjects) ; n = 31 [B] and n = 33 [D, F]. Data are shown as median and interquartile ranges. * indicates differences between young and older subjects. # indicates differences at baseline between young and older participants.

Figure 5. mRNA expression levels of *IL1β* [A, B], *IL10* [C, D] and *CCL5* [E, F] after an acute bout of strength exercise, expressed as fold changes, in young and older subjects in skeletal muscle and PBMC. Skeletal muscle (young people); n = 16 [C], n = 25 [A] and n = 30 [E]. Skeletal muscle (older subjects); n = 23 [C], n = 25 [E] and n = 26 [A]. PBMC (young subjects); n = 39 [D, F] and n = 40 [B]. PBMC (older subjects) ; n = 31 [B, F] and n = 33 [D].

Data are shown as median and interquartile ranges. * indicates differences between young and older subjects. # indicates differences at baseline between young and older participants.

Figure 6. Serum levels of IL6 in young (n=38) and older (n=32) subjects at baseline and one h after exercise. Data are shown as median and interquartile ranges.

1 Table 1

2

Amino acids (% of tot aa)	**Low-fat milk**	**WPC80**	**Native whey***
Alanine	3.2	4.8	4.6
Arginine	3.3	2.4	2.7
Aspartate	7.5	10.6	10.8
Cysteine	0.8	2.1	2.5
Phenylalanine	4.7	3.3	3.8
Glutamic acid	20.4	17.1	17.4
Glycine	1.9	1.9	1.9
Histidine	2.7	1.9	2.2
Isoleucine	4.9	6.0	5.3
Leucine	9.6	10.3	11.8
Lysine	8.2	9.2	9.9
Methionine	2.5	2.1	2.3
Proline	9.7	6.4	5.5
Serine	5.6	5.4	4.7
Threonine	4.4	7.0	4.9
Tyrosine	3.5	2.1	2.9
Valine	6.0	5.7	5.3
Tryptophan	1.3	1.7	2.0
EAA	41.4	45.3	45.2

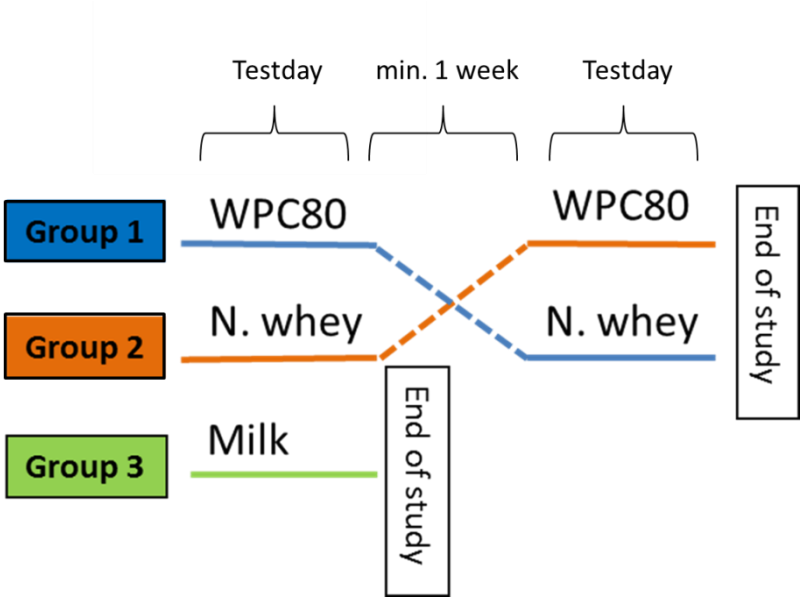
3 ** Mean values for native whey from study 1 and 2.*

Table 2 Baseline characteristics, independent on supplements provided

	Young (n=42)	Older (n=37)	p-value[1]
Age (yrs)	27.0 (25.4–28.6)	73.9 (72.7-75.1)	<0.001
Body mass (kg)	75.0 (70.9-79.2)	74.3 (69.9-78.7)	0.82
Lean mass (kg)	54.2 (50.9-57.5)	49.8 (46.4-53.1)	0.06
BMI (kg/m^2)	24.1 (23.1-25.2)	24.4 (24.4-25.6)	0.69
Fat percent (%)	24.9 (22.3-27.4)	29.3 (26.8-31.8)	0.02
Leg lean mass (kg)	18.9 (17.6-20.1)	17.1 (15.9-18.4)	0.05

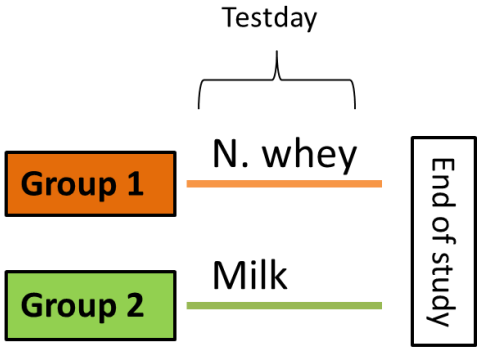
Values are expressed as means (95% confidence interval for mean). n: number of participants. BMI: body mass index. [1] Tested with Independent sample t-test

Figure 1a



WPC80; whey protein concentrate with 80 % protein, N.whey; native whey

Figure 1b



N.whey; native whey

Figure 2

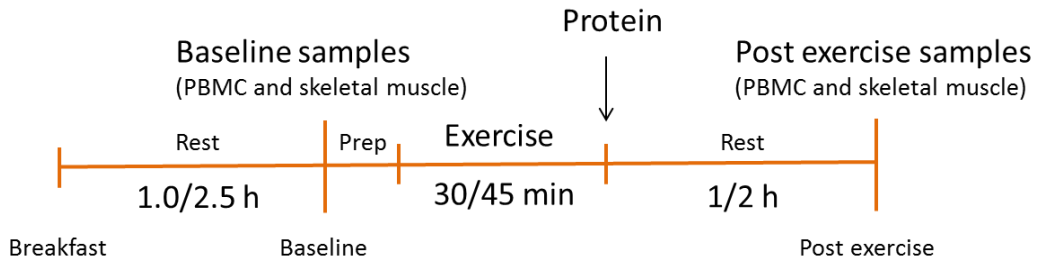

Protein
Baseline samples
(PBMC and skeletal muscle)
Post exercise samples
(PBMC and skeletal muscle)
Rest
Prep
Exercise
Rest
1.0/2.5 h
30/45 min
1/2 h
Breakfast
Baseline
Post exercise

Figure 3

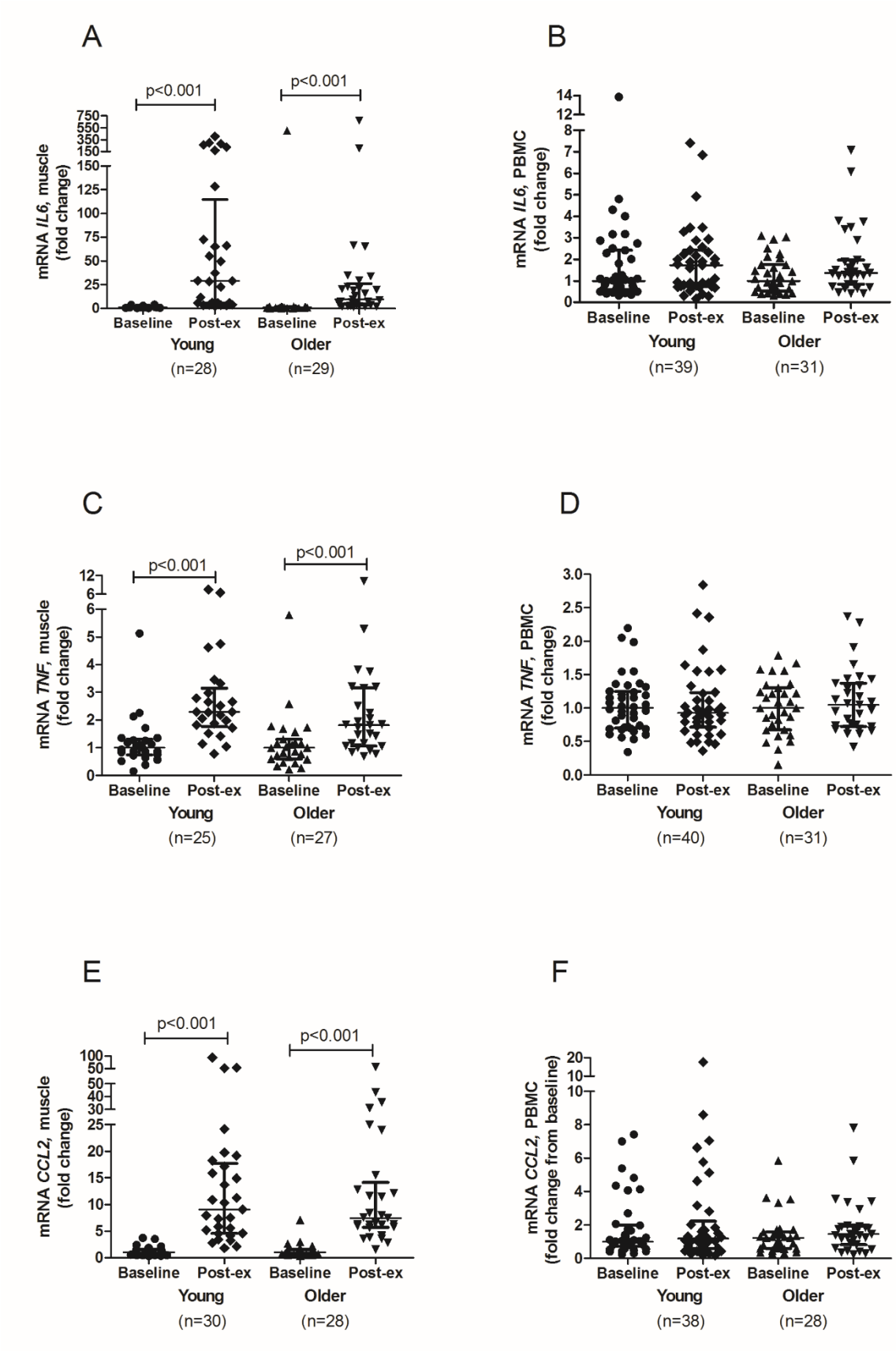

A
p<0.001
p<0.001
mRNA IL6, muscle (fold change)
750
550
350
150
150
125
100
75
50
25
0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=28)
(n=29)
B
mRNA IL6, PBMC (fold change)
14
12
8
7
6
5
4
3
2
1
0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=39)
(n=31)
C
p<0.001
p<0.001
mRNA TNF, muscle (fold change)
12
6
6
5
4
3
2
1
0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=25)
(n=27)
D
mRNA TNF, PBMC (fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=40)
(n=31)
E
p<0.001
p<0.001
mRNA CCL2, muscle (fold change)
100
50
50
40
30
25
20
15
10
5
0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=30)
(n=28)
F
mRNA CCL2, PBMC (fold change from baseline)
20
10
10
8
6
4
2
0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=38)
(n=28)

Figure 4

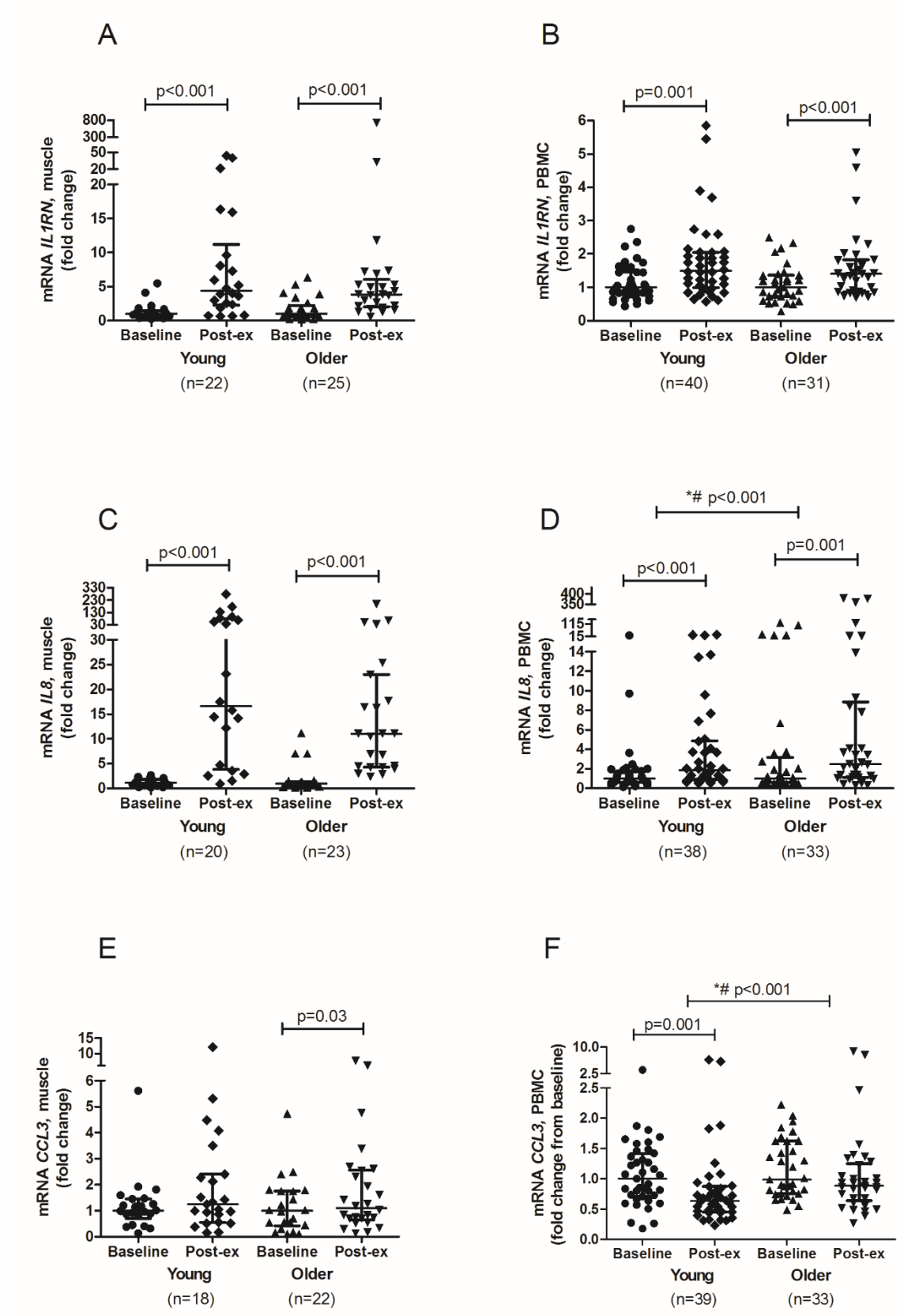

A
p<0.001
p<0.001
mRNA IL1RN, muscle (fold change)
800
300
50
20
20
15
10
5
0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=22)
(n=25)
B
p=0.001
p<0.001
mRNA IL1RN, PBMC (fold change)
6
5
4
3
2
1
0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=40)
(n=31)
C
p<0.001
p<0.001
mRNA IL8, muscle (fold change)
330
230
130
30
30
25
20
15
10
5
0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=20)
(n=23)
D
*# p<0.001
p=0.001
p<0.001
mRNA IL8, PBMC (fold change)
400
350
115
15
14
12
10
8
6
4
2
0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=38)
(n=33)
E
p=0.03
mRNA CCL3, muscle (fold change)
15
10
6
5
4
3
2
1
0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=18)
(n=22)
F
*# p<0.001
p=0.001
mRNA CCL3, PBMC (fold change from baseline)
10.0
2.5
2.5
2.0
1.5
1.0
0.5
0.0
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=39)
(n=33)

Figure 5

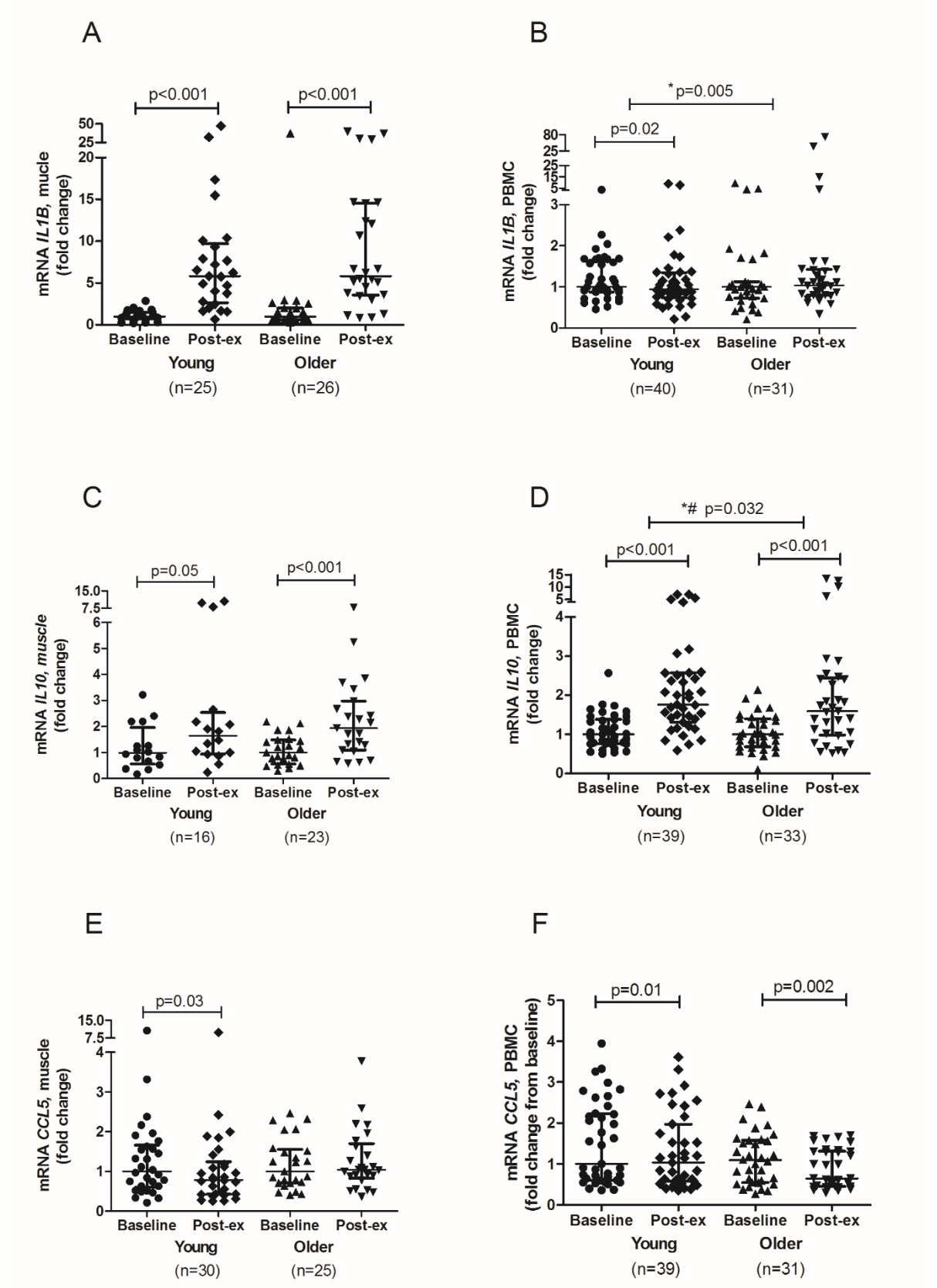
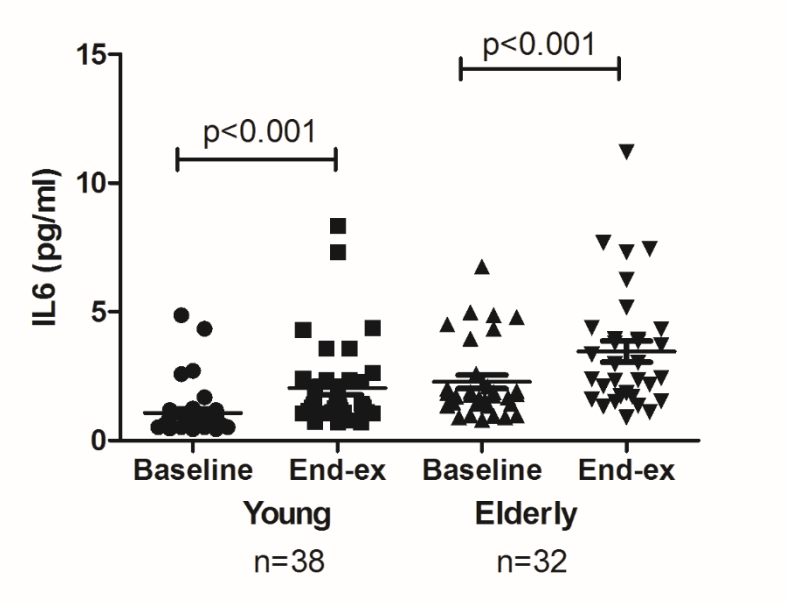

A
p<0.001
p<0.001
mRNA IL1B, mucle (fold change)
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=25)
(n=26)
B
*p=0.005
p=0.02
mRNA IL1B, PBMC (fold change)
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=40)
(n=31)
C
p=0.05
p<0.001
mRNA IL10, muscle (fold change)
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=16)
(n=23)
D
*# p=0.032
p<0.001
p<0.001
mRNA IL10, PBMC (fold change)
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=39)
(n=33)
E
p=0.03
mRNA CCL5, muscle (fold change)
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=30)
(n=25)
F
p=0.01
p=0.002
mRNA CCL5, PBMC (fold change from baseline)
Baseline
Post-ex
Baseline
Post-ex
Young
Older
(n=39)
(n=31)

Figure 6



n = number of participants

Additional file 1

Table A1 mRNA transcripts analyzed in both studies

Gene Symbol	Gene name	Entrez Gene ID (homo sapiens)	Assay ID	Function
ABCA1	ATP-binding cassette; sub-family A, member 1	19	Hs01059118_m1	Transporter involved in the regulation of cholesterol, involved in inflammation
CCL2	Chemokine (C-C-motif) ligand 2	6347	Hs00234140_m1	Involved in the acute response to exercise, important in chronic low-grade inflammation
CCL3	Chemokine (C-C-motif) ligand 3	6348	Hs00234142_m1	Involved in the acute inflammation by recruitment and activation of leukocytes.
CCL5	Chemokine (C-C-motif) ligand 5	6352	Hs00982282_m1	Involved in recruiting leukocytes to inflammatory sites.
CD36	CD36 molecule	948	Hs01567185_m1	Scavenger receptor involved in fatty acid metabolism
CXCL16	Chemokine (C-X-C-Motif) Ligand 16	58191	Hs00222859_m1	Involved in the migration of cells, a chemoattractant
IL10	Interleukin 10	3586	Hs00961622_m1	Down-regulates the expression of Th1 cytokines, enhances B cell survival, proliferation, and antibody production, able to block NF-κB activity
IL1β	Interleukin 1 beta	3553	Hs01555410_m1	Proliferation and maturation of lymphocytes, involved in inflammation and acute-phase response
IL1RN	Interleukin 1 receptor antagonist	3557	Hs00893626_m1	Inhibits the activities of IL1α/IL1β, and modulates a variety of interleukin 1 related immune and inflammatory responses
IL6	Interleukin 6	3569	Hs00985639_m1	A pleiotropic cytokine that plays important roles in the acute-phase response of exercise

				and in chronic low-grade inflammation
IL8	Interleukin 8	3576	Hs00174103_m1	Involved in angiogenesis in skeletal muscle, a chemoattractant
NR1H3	Nuclear receptor subfamily 1; group H; member 3	10062	Hs00172885_m1	Transcription factor, involved in lipid metabolism and inflammation
NR4A2	Nuclear receptor subfamily 4; group A; member 2	4929	Hs00428691_m1	Transcription factor, involved in energy metabolism and inflammation
NR4A3	Nuclear receptor subfamily 4; group A; member 3	8013	Hs00545009_g1	Transcription factor, involved in energy metabolism and inflammation.
PPARGC1A	Peroxisome proliferator-activated receptor gamma; coactivator 1 alpha	10891	Hs01016719_m1	Involved in energy metabolism and inflammation
TBP	TATA Box Binding Protein	6908	Hs00427620_m1	General transcription factor that functions at the core of the DNA-binding multiprotein factor TFIID (used as housekeeping gene)
TLR2	Toll-like receptor 2	7097	Hs01872448_s1	Involved in recognition of pathogen-associated molecular patterns, mediate the production of cytokines necessary for the development of effective immunity
TNF	Tumor Necrosis Factor	7124	Hs01113624_g1	Prototypical pro-inflammatory cytokine, play a central role in inflammation, immune system development and apoptosis

Additional file 2

Table A2 mRNA expression levels in PBMCs of young subjects

Gene	Timepoint	n (20-40 yrs)	$2^{-\Delta Ct}$ values (percentiles)			p-values (post exercise/ baseline)
			25	50	75	
ABCA1	Baseline	39	0.215	0.294	0.401	< 0.001
	Post exercise		0.275	0.399	0.553	
CCL2	Baseline	38	0.037	0.046	0.089	0.55
	Post exercise		0.029	0.052	0.096	
CCL3*#	Baseline	39	0.303	0.404	0.615	0.001
	Post exercise		0.196	0.277	0.382	
CCL5	Baseline	39	80.90	135.49	301.91	0.01
	Post exercise		79.15	135.08	259.32	
CD36	Baseline	41	7.46	9.24	12.20	< 0.001
	Post exercise		9.23	12.45	17.47	
CXCL16	Baseline	39	1.35	1.57	2.00	< 0.001
	Post exercise		1.77	2.25	3.12	
IL10	Baseline	39	0.010	0.013	0.019	< 0.001
	Post exercise		0.017	0.024	0.034	
IL1β	Baseline	40	0.552	0.630	1.042	0.02
	Post exercise		0.467	0.583	0.843	
IL1RN	Baseline	40	1.47	1.89	2.74	0.001
	Post exercise		1.81	2.83	3.88	
IL6	Baseline	39	0.005	0.008	0.019	0.11
	Post exercise		0.006	0.013	0.019	
IL8*#	Baseline	38	0.022	0.034	0.061	< 0.001
	Post exercise		0.031	0.061	0.153	
NR1H3	Baseline	38	0.245	0.434	0.698	0.01
	Post exercise		0.254	0.485	0.850	
NR4A2#	Baseline	40	0.030	0.041	0.060	0.47
	Post exercise		0.028	0.037	0.052	
NR4A3	Baseline	40	0.014	0.023	0.036	0.01
	Post exercise		0.014	0.026	0.045	
PPARGC1A*	Baseline	36	0.010	0.022	0.044	0.002
	Post exercise		0.023	0.038	0.069	
TLR2	Baseline	41	3.10	4.64	5.56	< 0.001
	Post exercise		4.77	6.29	8.37	
TNF	Baseline	40	1.07	1.52	1.90	0.80
	Post exercise		1.10	1.42	1.92	

n; number of participants. Values are calculated as $2^{-\Delta Ct}$, and shown at baseline and after exercise (post exercise). The mRNA expression levels were measured by quantitative real-time RT-PCR and normalized to the endogenous control TBP. p-values indicate changes

between post exercise and baseline values. * indicate differences between young and older participants at baseline. # indicate differences post exercise between young and older participants.

Table A3 mRNA expression levels in PBMCs of older subjects

Gene	Timepoint	n (≥ 70 yrs)	$2^{-\Delta Ct}$ values (percentiles)			p-values (post exercise/ baseline)
			25	50	75	
ABCA1	Baseline	30	0.198	0.306	0.394	0.02
	Post exercise		0.243	0.323	0.596	
CCL2	Baseline	28	0.031	0.053	0.086	0.06
	Post exercise		0.041	0.075	0.105	
CCL3*#	Baseline	33	0.442	0.590	0.960	0.18
	Post exercise		0.375	0.521	0.737	
CCL5	Baseline	31	115.44	187.143	342.31	0.002
	Post exercise		96.36	133.51	283.12	
CD36	Baseline	33	5.135	8.80	11.92	< 0.001
	Post exercise		9.48	13.03	18.26	
CXCL16	Baseline	33	1.39	1.87	2.50	0.001
	Post exercise		1.80	2.47	3.60	
IL10	Baseline	33	0.011	0.017	0.024	< 0.001
	Post exercise		0.017	0.027	0.041	
IL1β	Baseline	31	0.48	0.67	0.80	0.14
	Post exercise		0.53	0.68	0.94	
IL1RN	Baseline	31	1.38	1.89	2.59	< 0.001
	Post exercise		1.67	2.66	3.67	
IL6	Baseline	31	0.005	0.011	0.018	0.07
	Post exercise		0.008	0.013	0.022	
IL8*#	Baseline	33	0.035	0.061	0.200	0.001
	Post exercise		0.067	0.147	0.526	
NR1H3	Baseline	33	0.260	0.429	0.695	0.98
	Post exercise		0.242	0.327	0.736	
NR4A2#	Baseline	34	0.032	0.047	0.070	0.07
	Post exercise		0.034	0.058	0.086	
NR4A3	Baseline	34	0.018	0.023	0.033	0.46
	Post exercise		0.015	0.026	0.044	
PPARGC1A*	Baseline	32	0.008	0.020	0.039	0.98
	Post exercise		0.012	0.024	0.034	
TLR2	Baseline	34	2.87	4.12	5.24	< 0.001
	Post exercise		4.80	6.06	8.34	
TNF	Baseline	31	1.16	1.54	2.06	0.34
	Post exercise		1.11	1.61	2.11	

n; number of participants. Values are calculated as $2^{-\Delta Ct,}$ and shown at baseline and after exercise (post exercise). The mRNA expression levels were measured by quantitative real-time RT-PCR and normalized to the endogenous control TBP. p-values indicate changes between post exercise and baseline values. * indicate differences between young and older participants at baseline. # indicate differences post exercise between young and older participants

Table A4 mRNA expression levels in skeletal muscle of young subjects

Gene	Timepoint	n (20-40 yrs)	$2^{-\Delta Ct}$ values (percentiles)			p-values (post exercise/ baseline)
			25	50	75	
ABCA1	Baseline	27	1.3	1.59	2.05	0.46
	Post exercise		1.18	1.59	2.1	
CCL2	Baseline	30	0.23	0.34	0.55	< 0.001
	Post exercise		1.9	4.03	7.23	
CCL3	Baseline	18	0.006	0.014	0.021	0.27
	Post exercise		0.008	0.018	0.034	
CCL5[#]	Baseline	30	0.138	0.236	0.424	0.03
	Post exercise		0.113	0.206	0.318	
CD36	Baseline	28	24.76	40.07	44.09	0.04
	Post exercise		25.65	32.66	57.7	
CXCL16[*]	Baseline	30	0.085	0.125	0.153	< 0.001
	Post exercise		0.128	0.204	0.368	
IL10	Baseline	16	0.004	0.005	0.008	0.05
	Post exercise		0.005	0.008	0.012	
IL1β	Baseline	25	0.007	0.012	0.017	< 0.001
	Post exercise		0.024	0.068	0.094	
IL1RN	Baseline	22	0.007	0.013	0.018	< 0.001
	Post exercise		0.018	0.048	0.107	
IL6	Baseline	28	0.005	0.008	0.012	< 0.001
	Post exercise		0.046	0.238	1.055	
IL8	Baseline	20	0.006	0.01	0.016	< 0.001
	Post exercise		0.061	0.155	0.617	
NR1H3[*#]	Baseline	29	0.285	0.36	0.483	0.05
	Post exercise		0.348	0.391	0.497	
NR4A2[*#]	Baseline	29	0.032	0.052	0.074	< 0.001
	Post exercise		4.772	8.00	22.46	
NR4A3[*]	Baseline	29	0.28	0.37	0.64	< 0.001
	Post exercise		73.1	108.38	121.72	
PPARGC1A[*]	Baseline	30	6.32	7.26	8.68	< 0.001
	Post exercise		10.4	12.82	41.7	
TLR2	Baseline	29	0.06	0.08	0.12	0.001
	Post exercise		0.09	0.13	0.18	
TNF	Baseline	25	0.02	0.03	0.04	< 0.001
	Post exercise		0.06	0.08	0.11	

n; number of participants. Values are calculated as $2^{-\Delta Ct}$, and shown at baseline and after exercise (post exercise). The mRNA expression levels were measured by quantitative real-time RT-PCR and normalized to the endogenous control TBP. p-values indicate changes between post exercise and baseline values. * indicate differences between young and older participants at baseline. # indicate differences post exercise between young and older participants

Table A5 mRNA expression levels in skeletal muscle of older subjects

Gene	Timepoint	n (≥ 70 yrs)	$2^{-\Delta Ct}$ values (percentiles)			p-values (post exercise/ baseline)
			25	50	75	
ABCA1	Baseline	26	1.33	2.00	2.31	0.16
	Post exercise		1.26	1.71	2.82	
CCL2	Baseline	28	0.25	0.38	0.57	< 0.001
	Post exercise		2.13	2.64	5.57	
CCL3	Baseline	22	0.006	0.014	0.025	0.03
	Post exercise		0.01	0.014	0.035	
CCL5[#]	Baseline	25	0.175	0.264	0.454	0.66
	Post exercise		0.265	0.316	0.536	
CD36	Baseline	27	29.87	41.49	61.89	0.02
	Post exercise		31.63	46.75	75.11	
CXCL16[*]	Baseline	25	0.119	0.152	0.224	0.001
	Post exercise		0.151	0.221	0.311	
IL10	Baseline	23	0.003	0.006	0.009	< 0.001
	Post exercise		0.006	0.01	0.016	
IL1β	Baseline	26	0.005	0.008	0.017	< 0.001
	Post exercise		0.029	0.05	0.119	
IL1RN	Baseline	25	0.006	0.011	0.032	< 0.001
	Post exercise		0.022	0.043	0.061	
IL6	Baseline	29	0.006	0.012	0.02	< 0.001
	Post exercise		0.041	0.124	0.332	
IL8	Baseline	23	0.004	0.01	0.015	< 0.001
	Post exercise		0.044	0.113	0.254	
NR1H3[*#]	Baseline	28	0.385	0.458	0.599	0.06
	Post exercise		0.395	0.497	0.652	
NR4A2[*#]	Baseline	26	0.063	0.092	0.142	< 0.001
	Post exercise		1.14	2.442	9.845	
NR4A3[*]	Baseline	28	0.34	0.579	0.99	< 0.001
	Post exercise		44.10	98.98	122.49	
PPARGC1A[*]	Baseline	29	4.99	6.63	7.27	< 0.001
	Post exercise		10.34	21.48	58.79	
TLR2	Baseline	27	0.06	0.08	0.11	0.04
	Post exercise		0.08	0.1	0.14	
TNF	Baseline	27	0.02	0.04	0.06	< 0.001
	Post exercise		0.04	0.07	0.12	

n; number of participants. Values are calculated as $2^{-\Delta Ct}$, and shown at baseline and after exercise (post exercise). The mRNA expression levels were measured by quantitative real-time RT-PCR and normalized to the endogenous control TBP. p-values indicate changes between post exercise and baseline values. * indicate differences between young and older participants at baseline. # indicate differences post exercise between young and older participants

Paper IV

Gyrd O. Gjevestad, Håvard Hamarsland, Truls Raastad, Jacob J. Christensen, Anne S. Biong, Stine M. Ulven and Kirsten B. Holven

Eleven weeks of strength training decreased inflammatory markers in older subjects independent of protein supplement type; a randomized controlled trail. Submitted manuscript.