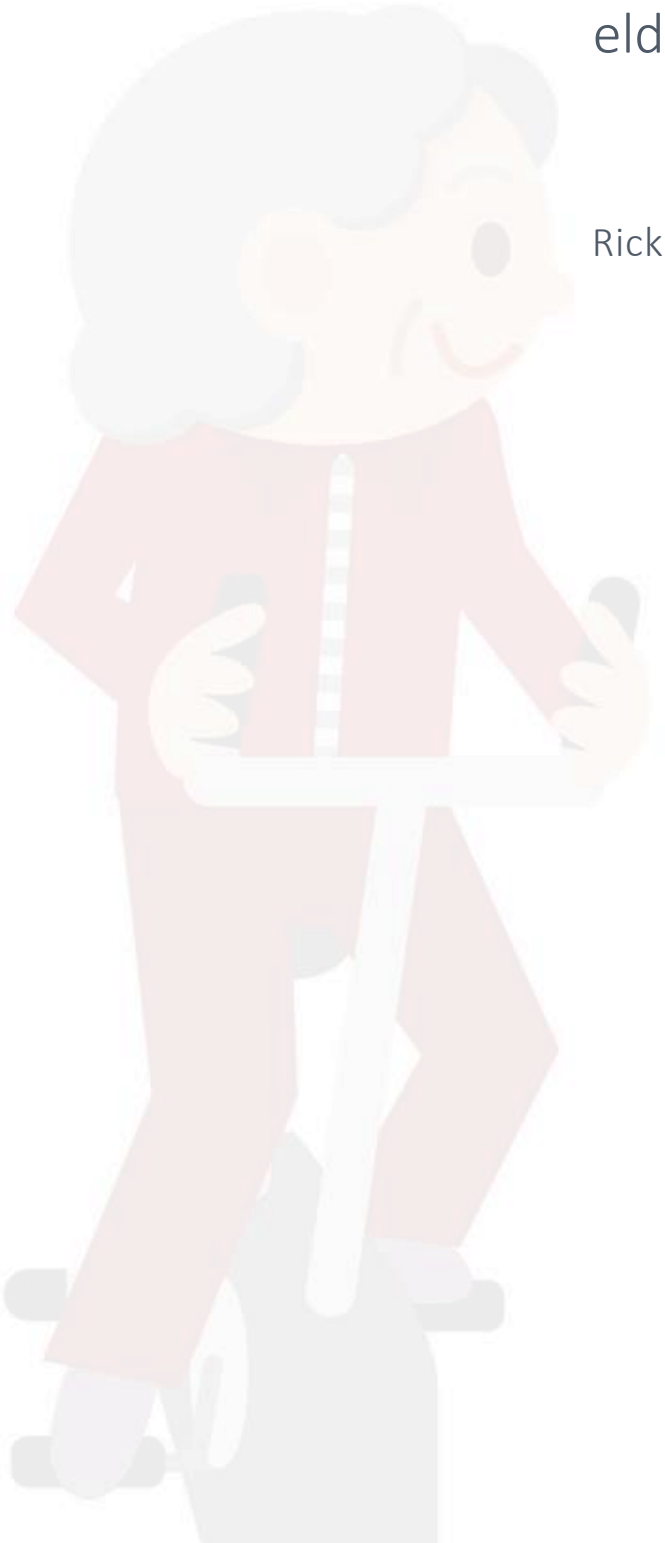
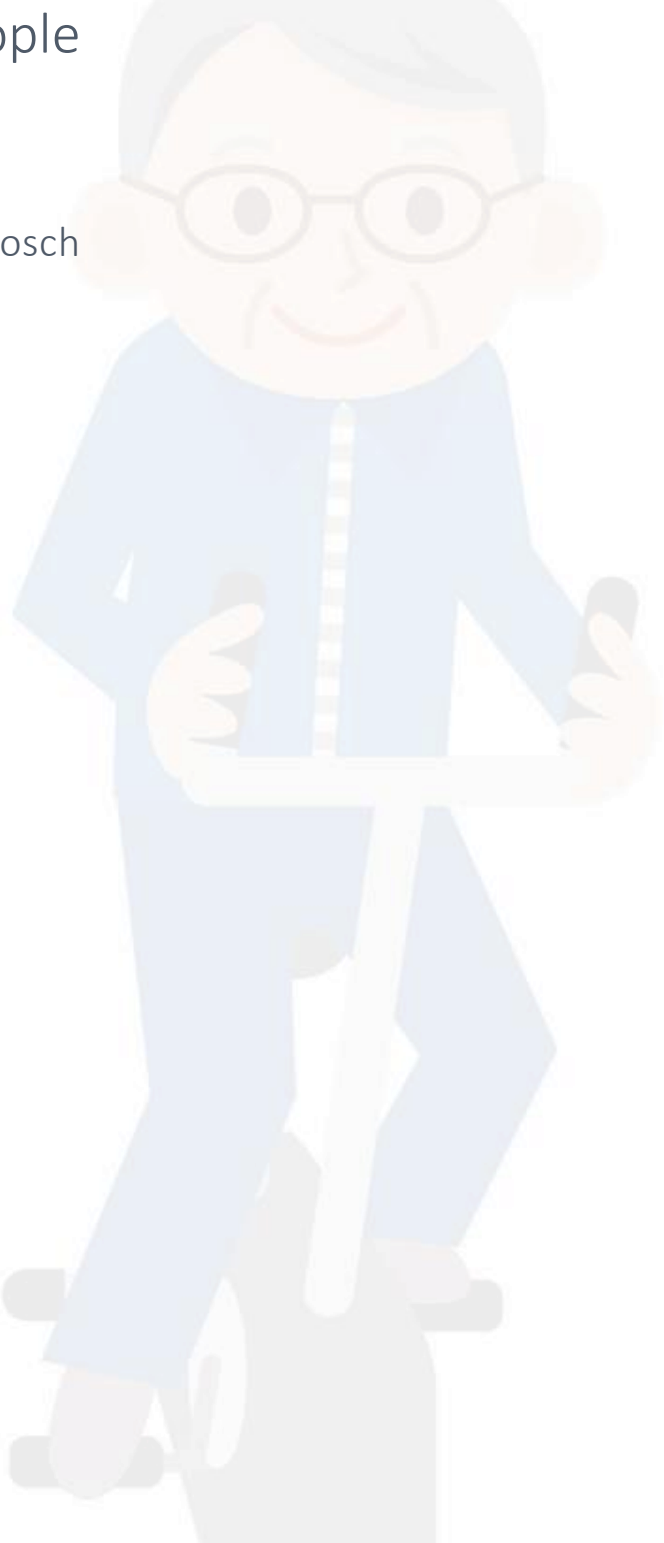
ProMuscle clinical trial:

effect of protein and exercise intervention on circulating levels of inflammatory cytokines in frail elderly people

Rick van den Bosch





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effect of protein and exercise intervention on circulating levels of inflammatory cytokines in frail elderly people

MSc Thesis

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Abstract

Background. As people get older they develop a state of systemic low level chronic inflammation. A higher inflammatory status is associated with different kinds of diseases such as CVD, dementia, frailty and sarcopenia. Epidemiologic studies show that exercise has an effect on the immune system and may have an anti-inflammatory effect. **Objectives.** The purpose of this study was to determine the effect of protein supplementation and resistance type exercise training on circulating blood levels of IL-1beta, IL-6, IL-8 and TNF-alpha, in frail elderly subjects. **Methods.** Data was collected from 2 randomized controlled trails of 6 months with (pre-) frail elderly (>65 years of age) subjects. In one study, subjects received daily protein supplementation or a placebo. In the other study subjects received daily protein supplementation or a placebo in combination with an exercise program. Thus making four groups; Protein-No exercise, Placebo-No exercise, Protein–Exercise and Placebo-Exercise. 65 frail and pre-frail elderly participated in the protein supplementation study without the exercise program, of whom 29 males. 62 frail and pre-frail elderly participated in in the protein study including the exercise program, of whom 21 males. The protein supplements contained 15 mg. protein in a 250 mL beverage and were ingested after breakfast and after lunch. The resistance type exercise program included two supervised sessions a week at 50-75% of 1-RM. Blood samples were taken at baseline, after 12 weeks and after 24 weeks to measure plasma levels of IL-1beta, IL-6, IL-8 and TNF-alpha. **Results.** The resistance type exercise training program did not have a statistically significant effect on plasma concentrations of TNF-alpha (p=0.661), IL-1beta (p=0.278), IL-6 (p=0.732) and IL-8 (p=0.404), following 24 weeks of intervention (p value for Treatment x Time interaction). Similarly there were no significant effects of protein supplementation on plasma levels of TNF-alpha (p=0.654), IL-1beta (p=0.275), IL-6 (p=0.627) and IL-8 (p=0.546). **Conclusion.** Neither protein supplementation nor an resistance-type exercise program results in detrimental or beneficial effects on the immune parameters of frail elderly subjects.

List of abbreviations

1-RM	1 Repetition Maximum
BMI	Body Mass Index
CVD	Cardiovascular Disease
EDTA	Ethylenediaminetetraacetic Acid
eGFR	estimated Glomerular Filtration Rate
IL	Interleukin
IL-1RA	Interleukin-1 Receptor Antagonist
ln	Natural Logarithm
RCT	Randomized Controlled Trail
RM	Repetition Maximum
SD	Standard Deviation
SEM	Standard Error of the Mean
sTNF-R	Soluble Tumor Necrosis Factor-Receptor
TNF	Tumor Necrosis Factor

1. Introduction

As a result of a higher life expectancy and the post war baby boomers the Dutch population is ageing rapidly. According to the population prognosis the elderly, above 65 years of age, will compose 26% of the population by 2040, compared to 16% in 2012 (Giesbers, Verweij, & Beer, 2013). The ageing of the population is of course accompanied by the increase of age related diseases, one of which is frailty. Characterized by an increased vulnerability to stressors and decreased physiologic reserves, frailty is a syndrome resulting in the deterioration of multiple physiological systems (Fried et al., 2004; Fried et al., 2001). Frailty in this way increases vulnerability to adverse health outcomes such as falls, morbidity, incident disability, hospitalization and mortality (Krabbe, Pedersen, & Bruunsgaard, 2004; Xue, 2011). It is also associated with major chronic diseases such as diabetes and cardiovascular diseases (Fried et al., 2004).

Fried et al. developed criteria by which frailty can be clinically identified. (1) unintentional weight loss, (2) self-reported exhaustion, (3) low grip strength, (4) slow walking speed and (5) low physical activity (Fried et al., 2001). When only one or two conditions are met, pre-frail is used as classification. With frailty being highly prevalent with increasing age, it is estimated that 38.5% of the Dutch population of over 65 years of age is pre-frail and 11.3% of the elderly is frail (Santos-Eggimann et al., 2009). A major not disease-based pathway by which elderly become frail is by ageing related decline of muscle mass and strength, called sarcopenia (Cruz-Jentoft et al., 2010; Fried et al., 2004). Sarcopenia can be caused by a combination of multiple factors including inadequate dietary protein intake, dysfunction of the neuroendocrine system and a sedentary lifestyle (Houston et al., 2008; Paddon-Jones & Rasmussen, 2009; Paddon-Jones et al., 2006; Roubenoff, 2000). Furthermore both Frailty and sarcopenia are strongly related to age-associated chronic low-grade inflammation, which contributes to muscle loss (Paddon-Jones & Rasmussen, 2009; Visser et al., 2002; Zoico & Roubenoff, 2002). And so, with regard to the ageing of the population and the increasing prevalence of frailty, it is important to understand the changing inflammatory milieu and to research the possibilities to beneficially alter it.

1.1 Cytokines

Cytokines are proteins secreted by immune mediating cells and which exert many different functions in adaptive and innate immunity responses. They can act locally as well as systemically. The latter usually occurs if the stimulus for production is so strong that large quantities are produced and enter the bloodstream and thus act as endocrine hormones at distant sites. Cytokines that are made by leucocytes and act on other leukocytes are called interleukins (Abbas & Lichtman, 2010). Increases in pro-inflammatory cytokines as a result of illness are part of the normal healing process. A sustained increase in plasma levels however does not assist in tissue repair and it does not promote the complement system, instead it leads to tissue damage and muscle loss (Addison et al., 2012). For example, TNF can act on the hypothalamus and induce fever. Prolonged production of TNF can induce appetite suppression and reduced synthesis of lipoprotein lipase, causing wasting of fat and muscle cells (Abbas & Lichtman, 2010). Ageing people are affected by such dysfunctions of the immune system. Advanced age is consequently associated with a decreased capacity to cope with stressors and a progressive increase in pro-inflammatory status referred to as inflamm-ageing (Franceschi et al., 2006). Chronic inflammation is one of the most important physiological causes to influence frailty in older adults and it can lead to reduced muscle strength, decreased mobility and sarcopenia (Addison et al., 2012). Plasma concentrations of inflammatory and anti-inflammatory mediators such as cytokines and acute phase proteins can increase by 2-4 fold as result of inflamm-ageing. These include pro-inflammatory cytokines like IL-6, IL-1beta and TNF-alpha (Krabbe et al., 2004).

With increasing age the immune system shows a decline in various ways. Decline is often visible in the formation of high-affinity antibodies, in the reduced generation of long lasting memory immune responses after vaccination, and in decreased expression of delayed type hyper sensitivity reactions to antigens (Miller, 1996). The adaptive immune function decreases primarily because of the decline in production of naive lymphocytes. This is a result of a reduction in thymic output of T cells, having less bone marrow, early progenitor B cells, and functionally incompetent memory lymphocytes. Ultimately this results in the decrease of the antigen recognitions repertoire (Weng, 2006). In addition to the decline in adaptive immune function there is also reason to suspect a negative effect on the innate system, especially in the non-healthy and frail (Gomez, Boehmer, & Kovacs, 2005). This might be due to a number of causes. For example, an impaired phagocytic function of macrophages, altered signal transduction pathways in neutrophils and phagocytes, decreased antigen presenting capacities and lower natural killer cell activity can have a negative effect on the innate immune system (Gomez et al., 2005; Solana, Pawelec, & Tarazona, 2006).

As a result of tissue damage due to an defective and overactive immune system, macrophages produce pro-inflammatory cytokines such as TNF-alpha and IL-1, which leads to the stimulation of the production of IL-6, recruitment of macrophages and induction of the acute phase protein synthesis (Addison et al., 2012). IL-6 has both pro- and anti-inflammatory effects. Increased IL-6 indirectly leads to the deregulation of TNF-alpha by stimulating the production of IL-10 and tumor necrosis factor soluble receptor, thus limiting the immune response (Addison et al., 2012; Zoico & Roubenoff, 2002). In addition IL-6 may play a role in the regulation of muscle proteolysis (Zoico & Roubenoff, 2002). The immune system has an effect on skeletal muscles, however skeletal muscles also affect the immune system. Several types of cells inside the muscles are able to produce cytokines. The main producers are neutrophils and macrophages, which produce among others IL-6, IL-8 and TNF-alpha. Other cells such as fibroblast, myocytes and endothelial cells are also able to produce cytokines in response to stimuli, like muscle contraction, shear stress and cytokines (Zoico & Roubenoff, 2002). Sources of IL-6 include adipose tissue, connective tissue, brain tissue (Pedersen, 2009), leucocytes and liver tissue (Gleeson et al., 2011).

The age related changes in the production of inflammatory mediators could be an effect of pre-existing conditions such as autoimmune diseases, cancer or other (subclinical) factors that lower the ability to fight infections. Furthermore a decreased production of sex hormones, relatively high body fat percentage and smoking are also mentioned to be related (Krabbe et al., 2004; Weng, 2006). Alternatively, it could be a result of age-associated defects in the immune system (Solana et al., 2006) or increased body fat percentage (Addison et al., 2012). Cause and effect are difficult to distinguish because a relatively large amount of studies conducted in the area did not include covariates like disease state, co morbidities and adipose tissue into the design (Addison et al., 2012). Low-level chronic inflammation could cause a negative effect or it could be a result of the associated diseases (Bruunsgaard, 2005). What can be said is that systemic low-level chronic inflammation is a predictor for all-cause mortality and cardiovascular disease-cause mortality in elderly (Bruunsgaard, 2005) furthermore it has been associated with, dementia, frailty and sarcopenia (Addison et al., 2012; Krabbe et al., 2004).

As was mentioned before sarcopenia is the decrease of muscle mass and strength associated with aging. Since muscle cells are able to produce cytokines, sarcopenia may have an effect on, or may be affected directly by chronic inflammation. Literature does not yet provide a consensus on this interaction. Decreased strength has several causes. It may be due to a combination of multiple factors, such as interruption in the excitation-concentration coupling process, the removal of actin and myosin proteins, a decrease in resting membrane potential of the sarcolemma, a disruption in calcium regulation (Addison et al., 2012) or a reduction in muscle fibers induced by denervation and impaired sprouting capacity (Aagaard et al., 2010). It appears also that cytokines, among others through proteolytic pathways, as regulatory molecules, have a part in muscle protein breakdown and muscle protein turnover in general (Zoico & Roubenoff, 2002). TNF-alpha seems to be the major contributor to strength and muscle mass impairment (Addison et al., 2012; Bruunsgaard, 2005).

1.2 Anti-inflammatory effects of exercise

Increased levels of pro inflammatory cytokines are troublesome for older adults. Chronic inflammation may, however, not be the result of old age in itself (Addison et al., 2012). Instead, activity levels may play a part. Elderly with higher activity levels show lower levels of inflammation (Addison et al., 2012; Fischer et al., 2007). There is evidence that the long term, regular physical activity can protect against the development of certain diseases like coronary heart diseases, strokes, cancer and type 2 diabetes (Gleeson et al., 2011; Mathur & Pedersen, 2008; Petersen & Pedersen, 2005). It is therefore not surprising that observational studies link physical exercise to an anti-inflammatory effect.

The levels of systemic cytokines that exercise induces are similar to the levels of infections. A difference is that TNF-alpha and IL-1beta do not increase due to exercise when no muscle damage is caused (Bruunsgaard, 2005). This indicates that the cytokine response pathway to exercise is different than the acute phase response induced by infections (Bruunsgaard, 2005; Petersen & Pedersen, 2005). During exercise IL-6 is produced by myocytes, presumably resulting in an increase in anti-inflammatory cytokines and a decrease in pro-inflammatory cytokines (Addison et al., 2012). Cytokines such as IL-6, IL-1beta and TNF-alpha temporarily increase during and directly after strenuous exercise. IL-6 may even increase up to a hundred times, making this cytokine the most prominent responder to exercise (Kasapis & Thompson, 2005). Concentrations return to pre-exercise levels after 2.5 hours, except for IL-6 which remains significantly higher than pre-exercise levels after 4 hours. Anti-inflammatory IL-10 and cytokine inhibitors IL-1ra, sTNF-r1 and sTNF-r2 balance the release of the pro-inflammatory cytokines and reach peak concentrations about one hour after exercise (Ostrowski et al., 1999).

The mechanisms by which exercise has an anti-inflammatory effect are not yet fully understood. One theory is that IL-6 response is brought on by exercise-induced muscle damage. Muscle damage triggers a repair response in which macrophages enter the muscle and contribute to IL-6 production (Kasapis & Thompson, 2005). Concentric and eccentric muscle contractions appeared to induce a different IL-6 response due to the extra damage eccentric muscle contractions cause (Kasapis & Thompson, 2005; Ostrowski et al., 1999). However the more current consensus is that IL-6 is mostly released by muscles independently of muscle damage (Febbraio & Pedersen, 2002; Petersen & Pedersen, 2005). The IL-6 production associated with muscle damage is more delayed and of smaller magnitude (Kasapis & Thompson, 2005; Ostrowski et al., 1999). Furthermore eccentric exercise compared to concentric exercise does not lead to a higher IL-6 response. This indicates that muscle damage per se is not required to increase plasma levels of IL-6 (Pedersen & Febbraio, 2008). Instead, exercise intensity, duration and used muscle mass are directly related to levels of IL-6 (Febbraio & Pedersen, 2002). Pedersen et al state that exercise duration is the most important factor affecting plasma IL-6 increase. Up to 50% of the variation of IL-6 levels can be explained by the duration of the exercise. Furthermore, active muscle mass is said to be of a determining factor for fluctuation in plasma IL-6 levels. Running for example, involves more contracting muscles than exercise with upper extremities and should therefore induce a bigger fluctuation (Mathur & Pedersen, 2008).

Multiple factors are related to the anti-inflammatory effect of exercise. In addition to an improved blood lipid profile and increased cardiovascular health it is possible that beneficial effects are induced by exercise in itself (Gleeson et al., 2011; Kraus et al., 2002). Gleeson et al. give three possible mechanisms by which exercise can induce anti-inflammatory effects. The first is the reduction of visceral fat mass. Excessive fat mass is linked to increased levels of pro-inflammatory cytokines TNF-alpha, IL-6 and C-reactive protein in elderly (Addison et al., 2012). Adipocytes gain weight and increase in size because of increased fat levels in the body, which can cause adipocytes to become dysfunctional due to increased levels of lipids in the cell and hypoxia within the adipocyte itself. Eventually this can lead to apoptosis of the adipocyte, which induces an inflammatory response (Schenk, Saberi, & Olefsky, 2008). local pro-inflammatory cytokine levels increase and macrophages are recruited, which itself also secrete cytokines, to attract more macrophages.

If the scale of this cycle is big enough it will lead to chronic inflammation (Schenk et al., 2008; Yudkin, 2007). The reduction of fat mass by means of exercise results in a smaller inflammatory response and therefore might lead to lower systemic inflammation (Gleeson et al., 2011)

In addition the release of IL-6 from contracting muscle is given as possible explanation for the anti-inflammatory effects of exercise (Gleeson et al., 2011). Exercise induced muscle contraction contributes to higher plasma levels of IL-6 by ways of a spillover effect (Steensberg et al., 2000). This increase in IL-6 leads to increasing levels of IL-10 and IL-1RA and cortisol release, producing an anti-inflammatory environment (Petersen & Pedersen, 2005). IL-1RA inhibits pro-inflammatory actions of IL-1beta. IL-10 down regulates several pro-inflammatory cytokines and mediators (Gleeson et al., 2011). The authors indicate that the cascade of actions, that muscle derived IL-6 is responsible for, do not account for all the health benefits on its own, as low to moderate exercise does not induce this response (Fischer et al., 2006). Thirdly, increased levels of circulating cortisol and adrenaline are introduced as an in exercise induced anti-inflammatory effect. Exercise induces an increased secretion of cortisol and catecholamines by the hypothalamus and sympathetic nervous system (Gleeson et al., 2011). Cortisol is said to have an anti-inflammatory effect and catecholamines down regulate the production of TNF-alpha and IL-1beta by immune cells (Petersen & Pedersen, 2005). Furthermore cortisol is mentioned to have an increasing effect on IL-6 production of skeletal muscle (Gleeson et al., 2011).

1.3 Objectives

Although the mechanisms behind the presumably anti-inflammatory effect of exercise are not yet totally understood it is clearer that physical activity has an effect on inflammation markers and it is known that acute bouts of exercise increase concentrations of pro-inflammatory cytokines (Ostrowski et al., 1999; Pedersen et al., 1998). Chronic low-grade inflammation is associated with multiple diseases, and therefore regular exercise might have a protective effect against the medical disorders that are associated with systemic low grade inflammation (Petersen & Pedersen, 2005). Therefore this study is conducted with the hypothesis that resistance type exercise causes a reduction in the plasma levels of IL-1beta, IL-6, IL-8 and TNF-alpha. With regard to the ageing of the population and the increasing prevalence of frailty, it is important to understand the changing inflammatory milieu and to research the possibilities to beneficially alter it. This study measures the effect of resistance type exercise and protein supplementation on plasma levels of the inflammatory cytokines IL-1beta, IL-6, IL8 and TNF-alpha in frail elderly men and women.

2. Methods

2.1 Study design

The findings used in this study were collected from two studies conducted by the Division of Human Nutrition from Wageningen University. Both are 24 week double blind randomized controlled trials conducted with a total of 127 frail and pre-frail elderly men and women. The aim of both studies was to determine the impact of dietary protein supplementation on muscle mass, strength and physical performance. They were similar in design except for the intervention. In one study, subjects received daily protein supplementation or a placebo. In the other study subjects received daily protein supplementation or a placebo in combination with an exercise program. The design and main findings of both studies were published (Tieland et al., 2012a; Tieland et al., 2012b). Subjects gave their written consent and the Wageningen University Medical Ethical Committee approved the study.

2.2 Study participants

Detailed selection criteria and the screening procedure can be found in the published articles. In short, the study population consisted of frail or pre-frail elderly subjects (>65 years old). Both men and women were included. Frailty was assessed using the five Fried criteria (Linda P Fried et al., 2001). When one or two criteria were met the subject was classified as pre-frail. When three or more criteria were met the subject was classified as frail. Subjects who were diagnosed with cancer, chronic obstructive pulmonary disease, type 2 diabetes, renal insufficiency, muscle disease or subjects who were unable to perform the exercise regimen were excluded from the study. Table 1 displays the subject characteristics per intervention group.

Table 1
Subject Characteristics

Variable	No exercise-Placebo(n=31)	Exercise-Placebo(n=31)	No exercise-Protein(n=34)	Exercise-Protein(n=30)
Age, y	81±1	79.2±1.1	78±1	78±2
Female/Male, n	16/15	21/10	20/14	19/11
Weight, kg	73.8±2.1	77.4±2.4	73.9±2.4	80.4±2.8
Height, m	1.67±0.02	1.66±0.02	1.65±0.02	1.66±0.02
BMI, kg/m2	26.2±0.6	28.2±0.8	27.0±0.8	28.9±0.8

Mean ± SEM
BMI = Body Mass Index, n = number of subjects, SEM = Standard Error of the Mean

2.3 Interventions

After inclusion, subjects were randomly allocated to the protein or to the placebo group, either with or without a 24-week resistance type exercise training program. This gives the resulting four groups: No exercise - Placebo, Exercise - Placebo, No exercise-Protein and the Exercise-Protein group.
The subjects who received protein supplementation either received a 250-mL beverage containing 15g protein or a matching 250mL placebo containing no protein. All beverages were packed in non-transparent packages and vanilla flavoured, to be consumed after breakfast and after lunch for the duration of the study.
The physical exercise intervention program consisted of a resistance type exercise training program conducted under supervision for two times a week over a period of 24 weeks. Training started with a 5 minute warm up on a cycle ergometer. Warm up was followed by 4 sets of 10 to 15 repetitions on leg-press and leg-extension machines. After this were 3 sets of 10-15 repetitions on chest press, lat pull-down, pec-dec, and vertical row machines.
The workload was set at 50-75% of 1-RM (1 repetition maximum). 1-RM was recalculated after 4,8,12,16 and 20 weeks of training, the intensity of the training was adjusted accordingly.

2.4 Measurements

Blood samples were taken at baseline, at 12 weeks and at 24 weeks of intervention. After an overnight fast, blood samples were collected in EDTA-containing tubes and centrifuged for 10 minutes at 4°C prior to storage at -80°C. The samples were analysed at the Department of Internal Medicine, Maastricht University to determine IL-1beta, IL-6, IL-8 and TNF-alpha concentrations.

2.5 Statistical analysis

Data analysis was performed with IBM SPSS statistics version 19. An independent Student t-test was used to compare baseline characteristics between treatment groups. Differences between treatments over time were analysed using mixed linear models. Time, Treatment, Age and the interaction between Time and Treatment were considered fixed factors and subjects were considered a random factor. With use of likelihood ratio tests it was determined that Gender and BMI did not contribute to the model and were therefore left out. To best approximate the conditional normality assumption, IL-1beta, IL-6, IL-8 and TNF-alpha values were transformed into natural logarithms for the analysis. For all values data is presented in mean ± standard error of the mean (SEM). p values less than 0.05 were considered statistically significant.

3. Results

The 24 week resistance type exercise training program did not have a statistical significant effect on plasma concentrations of TNF-alpha (p=0.661), IL-1beta (p=0.278), IL-6 (p=0.732) and IL-8 (p=0.404). There were no significant effects of protein supplementation on plasma levels of TNF-alpha (p=0.654), IL-1beta (p=0.275), IL-6 (p=0.627) and IL-8 (p=0.546). No changes within groups were observed. Also not within the IL-8 Exercise – Placebo group (p=0.195). This is in contrast to the original main intervention were effects of protein supplementation and the resistance-type exercise were found. Protein supplementation as well as resistance type exercise training improved physical performance. Additionally, protein supplementation also increased lean body mass, and resistance type exercise training improved strength over a period of 24 weeks.
Table 2 shows the plasma levels of IL-1beta, IL-6, IL-8 and TNF-alpha at baseline (T0), half-way (T3) and endpoint (T6) for each group. Values are displayed in mean ± SEM. Furthermore p-values for treatment x time interaction are given.

Table 2
Cytokine concentrations for all groups at 3 and 6 months from baseline

	No exercise-Placebo			No exercise-Protein			Exercise-Placebo			Exercise-Protein			p value Treatment x Time	
													Exercise	Supplementation
Variable	T0	T3	T6	T0	T3	T6	T0	T3	T6	T0	T3	T6		
IL-1beta (pg/ml)	0.4±0.1	0.4±0.0	0.4±0.0	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.0	0.5±0.1	0.5±0.1	0.4±0.1	0.4±0.0	0.4±0.0	0.28	0.28
IL-6 (pg/ml)	2.1±0.2	2.4±0.3	2.8±0.6	2.5±0.4	2.1±0.3	2.3±0.4	2.7±0.5	5.2±3.2	2.3±0.3	2.0±0.2	6.0±3.9	2.7±0.5	0.73	0.63
IL-8 (pg/ml)	19.7±11.4	18.2±9.2	19.0±9.5	10.4±2.1	11.0±2.3	11.2±2.1	8.5±0.4	9.9±0.7	9.8±0.7	9.0±0.7	9.5±0.6	10.0±0.9	0.4	0.55
TNF-alpha (pg/ml)	4.5±0.4	4.6±0.4	4.5±0.4	4.4±0.3	4.3±0.3	4.4±0.4	4.6±0.5	4.8±0.5	4.3±0.4	4.1±0.2	4.1±0.2	4.3±0.3	0.66	0.65

Mean ± SEM

Plasma cytokine levels did not differ among the groups at baseline. However, IL-8 levels of the No exercise – Placebo group catches the eye. Mean values are almost double compared to other groups, although not statistically significant (p=0.47). Seeing the SD of 62.5, high variability among the values is present. One extreme outlier of 350 pg/mL raises the average at baseline. The same subject is responsible for the other extreme outliers at T3 and T6. With a median value of 8.26 pg/mL the IL-8 No exercise – Placebo group is comparable to the Exercise – Placebo group with 8.04 pg/mL.

Notable in the results is the relatively big variance between subjects for the cytokine plasma levels as shown in Figure 1. The figure shows boxplots for baseline, T3 and T6 cytokine concentration of all subjects per cytokine. To best suit the conditional normality assumption for the mixed linear model analysis the values are transformed using natural logarithms. Despite the ln transformation, there are many outliers and even extreme outliers within all groups. 22 different subjects are responsible for all the outlying values. 9 subjects produce outlying values on all 3 time points and 5 produce outliers for more than one cytokine. Although the sample size is relatively large, the extreme values have a big influence on the means. They also cause the large standard deviations as can be seen in table 3. Especially high values are found for IL-6 and IL-8 levels.

Table 3

Cytokine concentrations of all subjects

	n	Minimum	Maximum	Mean	Std. Deviation	Variance
IL-1beta (pg/mL)	352	0.05	3.17	0.42	0.32	0.10
IL-6 (pg/mL)	352	0.44	114.62	2.92	7.94	63.11
IL-8 (pg/mL)	352	2.27	350.26	12.14	27.78	771.82
TNF-alpha (pg/mL)	352	2.18	15.12	4.40	1.93	3.74

n = total number of measurements, includes measurements of every subject at T0, T3 and T6

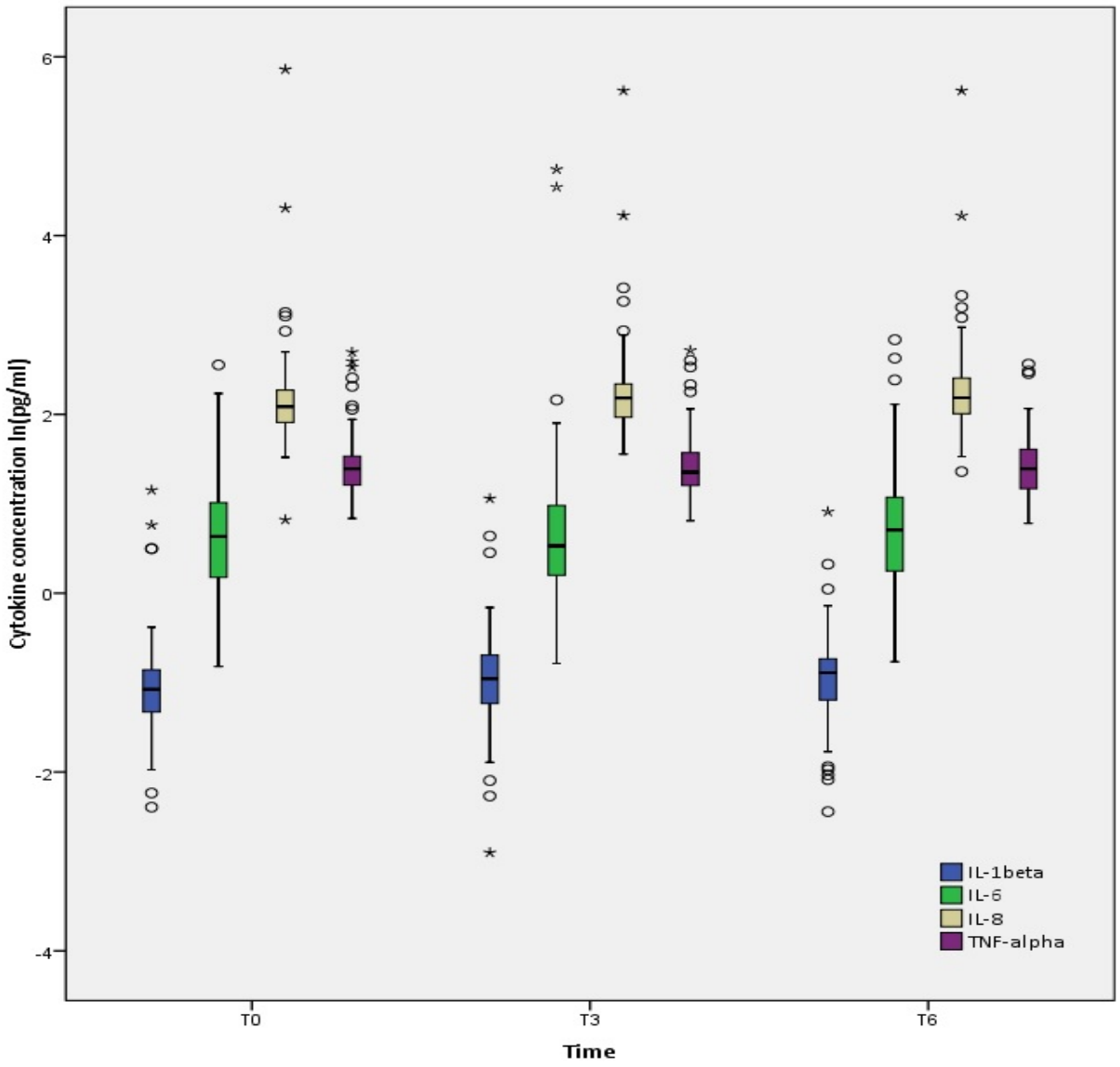

Figure 1

Boxplots of IL-1beta, IL-6, IL-8 and TNF-alpha ln transfored concentrations of all subjects.
o and * are outliers and extreme outliers.

Table 4 and 5 show the same information as table 2 and 3 but only for subjects with cytokine concentrations that fall within the range of mean ± 2 standard deviations.

Table 4

Cytokine concentration of all subjects after excluding values greater than mean ± 2 SD

	n	Minimum	Maximum	Mean	Std. Deviation	Variance
IL-1beta (pg/ml)	343	0.05	1.05	0.38	0.14	0.02
IL-6 (pg/ml)	350	0.44	17.04	2.34	1.91	3.65
IL-8 (pg/ml)	346	2.27	30.42	9.13	3.42	11.70
TNF-alpha (pg/ml)	339	2.18	8.14	4.10	1.14	1.30

n = total number of measurements, includes measurements of every subject at T0, T3 and T6

Table 5

Cytokine concentrations for all groups at 3 and 6 months from baseline after excluding values greater than mean ± 2 SD

	No exercise-Placebo			No exercise-Protein			Exercise-Placebo			Exercise-Protein			p value Treatment x Time	
													Exercise	Supplementation
Variable	T0	T3	T6	T0	T3	T6	T0	T3	T6	T0	T3	T6		
IL-1beta (pg/ml)	0.4±0.0	0.4±0.0	0.4±0.0	0.3±0.0	0.3±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.3±0.0	0.4±0.0	0.4±0.0	0.28	0.25
IL-6 (pg/ml)	2.0±0.2	2.4±0.3	2.8±0.6	2.5±0.4	2.1±0.3	2.3±0.3	2.7±0.4	2.0±0.2	2.3±0.3	2.0±0.2	2.1±0.3	2.7±0.5	0.86	0.73
IL-8 (pg/ml)	8.3±0.3	9.0±0.4	9.4±0.5	8.4±0.6	8.8±0.9	9.3±0.7	8.5±0.4	9.9±0.7	9.8±0.7	9.0±0.7	9.5±0.6	10.0±0.9	0.56	0.52
TNF-alpha (pg/ml)	4.0±0.2	4.1±0.2	4.2±0.3	4.2±0.2	4.1±0.2	4.1±0.2	4.0±0.2	4.1±0.2	4.0±0.2	4.1±0.2	4.1±0.2	4.3±0.3	0.87	0.86

Mean ± SEM

4. Discussion

This study aimed to determine the impact of resistance type exercise training and protein supplementation on plasma levels of IL-1beta IL-6, IL-8, and TNF-alpha in frail elderly subjects. The results show that after 24 weeks neither resistance-type exercise training nor protein supplementation had an effect on the plasma cytokine levels. Therefore the hypothesis that exercise reduces systemic low-grade inflammation is rejected. This conclusion is shared by multiple other similar studies.

4.1 Resistance-type exercise

Very recently Wanderley et al. conducted a randomized controlled trail with 50 Caucasian elderly. Three groups were made, one group as controls and two groups with training protocols. Both groups trained three time a week for eight months for 50 minutes per day. One group did aerobic training sessions with low intensity exercises, like walking and cycling at 50-70% of heart rate reserve. The other group had a resistance exercise protocol covering all major muscle groups, which included leg press and leg extension exercises with two sets of about 13 repetitions at 50-60% of 1-RM. The measured IL-6 and TNF-alpha levels did not differ in either training protocol. Rather they suggest that, next to a small sample size, the training protocols avoided an increase of cytokine plasma levels due to the significant increase in TNF-alpha in the control group. A large inter subject variation is mentioned to temper their conclusion (Wanderley et al., 2013).

In another RCT So et al. conducted a 12 week elastic band exercise program with 40 male and female Korean elderly subjects. The exercise program was performed three times per week with bouts of about sixty minutes and 2-3 sets of 15 repetitions. The exercise intensity was relatively low and included leg press and seated-row among others. Coherent with the findings of Wanderley et al. and our study, plasma levels of TNF-alpha, IL-6 and IL-1beta did not differ between intervention and control groups. Again, intra subject variation is mentioned as a limitation (So et al., 2013).

Lucosta et al. conducted a RCT over a period of 10 weeks with 32 pre-frail female elderly subjects. The resistance-type exercise training program consisted of 10 exercises at 75% of 1-RM including knee extension with one hour sessions for 3 times a week. IL-6 levels did not significantly differ between groups. The authors suggest that the duration of the program might have influenced the result. The 10 week duration may not have been sufficient to detect changes because the intercellular changes may not yet be pronounced enough to detect. Also they point at a small sample size (Lustosa et al., 2013). Another relatively short study was conducted by Stewart et al. After a 12 week, three day a week, resistance training exercise program with 32 elderly subjects the authors concluded that there were no significant intervention effects on plasma IL-6, IL-1beta and TNF-alpha concentrations among these elderly (Stewart et al., 2007). The same limitations applied to Bruunsgaard et al who conducted a RCT of 12 weeks with 21 frail elderly subjects. Three resistance-type exercise training sessions of approximately 45 minutes were done every week. Plasma levels of TNF-alpha and IL-6 did not change over time. The authors discuss if the duration of the program and the number of subjects were sufficient to detect an effect (Bruunsgaard et al., 2004).

The previous studies all suggest the expected change in plasma cytokine levels failed to occur due to a limited number of subjects or a limited duration of the intervention. Our study is able to show that a longer duration and a greater number of subjects also yields no different results. Furthermore there are other studies with even more subjects and longer durations that share the same results. These are, however, less similar in design as the others. Whereas the above mentioned studies have a similar exercise intensity with programs of about one hour for three times a week, Kaspasi et al. implements an exercise intervention four times a day. This RCT combined both resistance and endurance training in a group of 190 frail elderly nursing home subjects. Since they are elderly from a nursing home, resistance and endurance training have, compared to the other studies, a different intensity in the form of arm curls, arm raises and walking.

Nonetheless the exercises were done at 75% of the subjects' 1-RM. Levels of sTNF-RII were measured. In this 32 week RCT the authors assume that sTNF-RII reflects TNF-alpha activity. The intervention did not have a significant effect on sTNF-RII plasma levels (Kapasi et al., 2003).

Friedenreich et al. also did have a large sample size (n=320) and longer duration (12 months) for their RCT. The subjects however cannot be classified as elderly, these postmenstrual women had an average age of about 61 years. The exercise program consisted of aerobic exercise for five days a week with a session of 45 minutes at 70-80% heart rate reserve, but this did not cause change in plasma levels of IL-6 and TNF-alpha (Friedenreich et al., 2012).

4.1.1 Dissimilar results

There are few studies with similar designs that did find an effect of resistance type exercise on cytokine plasma levels. In a relatively long RCT of 12 months, 424 elderly men and women participated in the Lifestyle Interventions and Independence For Elders trial (LIFE). This RCT with a duration of 12 months included a physical activity program consisting of a combination of moderate intensity aerobics-, strength-, balance- and flexibility exercises of 40-60 min for 3 times a week. The physical activity intervention significantly lowered plasma IL-6 levels compared to the control group (Nicklas et al., 2008). Furthermore Greiwe et al. found with a small group of elderly (n=13) that resistance exercise training for three months caused lower levels of TNF-alpha. TNF-alpha levels were however measured from skeletal muscle biopsies, which is different from the other conducted studies (Greiwe et al., 2001). Another study that also found a reduction of TNF-alpha plasma levels after resistance type exercise included 30 elderly who were randomly put in a 12 week program in either the low resistance training group (8-11 repetitions in 2-4 sets at 40% of 1-RM) or in the high resistance training group (8-11 repetitions in 2-4 sets at80% of 1-RM). Exercises included leg extension and leg press and were done three sessions a week, of those one was supervised in the low resistance group and two in the high resistance group. There were no differences between groups for IL-6 and TNF-alpha. Only a significant reduction of TNF-alpha in the low resistance exercise group was found (Onambélé-Pearson, Breen, & Stewart, 2010a). Seeing this reduction happened within group and not relative to a control group, this result has less weight.

It seems plausible that regular exercise lowers plasma markers of inflammation. However practice shows less clear results than theory suggests. Some studies find a change but most do not. It has been suggested that a smaller sample size and shorter intervention period possibly inhibit the detection of an otherwise ongoing change of plasma cytokine levels. Studies that do find an effect are more scarce and are less convincing due to their more divergent research design. Greiwe et al had a very small sample size and Onambélé-Pearson et al did not have a true control group. Only Nicklas et al. with their LIFE study had a long duration and a large sample size. It could be possible that the levels of other inflammatory markers change in elderly people in reaction to resistance type exercise, however IL-1beta, IL-, IL-8 and TNF-alpha do not change, as was proven in our research.

4.2 Protein supplementation

The results of this study are derived from a RCT that aimed on determining the impact of dietary protein supplementation on muscle mass, strength and physical performance. There is a lot of literature available regarding the relations between protein supplementation and muscles. Nowadays the connection between the two is obvious. However, this cannot be said about protein supplementation and plasma cytokine levels. The data of the original study gives an unique opportunity to explore this relationship. This current study did not find an effect of protein supplementation on the levels of IL-1beta, IL-6, Il-8 and TNF-alpha in frail elderly people. There is little data to compare the results with since little research has been done on the influence of protein supplementation on plasma cytokine levels, especially not in elderly people. Furthermore protein supplementation is a very broad term.

There are many different proteins and there is no standard protein supplement so protein supplements do not only vary amongst each other in in dosage but also in composition. Protein supplements often do not consist solely out of protein. Proteins are administered in a supplement with additional ingredients for practical reasons.

Due to the scarce amount of literature available the following studies that will be discussed resemble this current study less than the previous discussed studies with resistance type exercise training. They all have some form of protein supplement for older people.
In a RCT with 31 postmenstrual, but not elderly, women soymilk supplementation was used to test if it would improve systemic markers of inflammation. The trial lasted for four weeks and a soymilk group was compared to a dairy control group. The trial included a 2 week run in phase in which participants were asked to limit the intake of dairy products and to completely stop using dairy milk. Both the Soymilk and dairy milk supplementation did not result in a change in plasma levels of TNF-alpha, IL-1beta and IL-6 (Beavers et al., 2009). Interesting to see is that isolated compounds from dairy milk, when supplemented, yield different results. In research by Bharadwaj et al. the milk protein lactoferrin enriched with ribonuclease was supplemented in a 6 month study. The subjects were similar, 38 post-menstrual not elderly women. Lactoferrin was supplemented with a total of 250 mg. a day. Compared to placebo IL-6 and TNF-alpha levels lowered significantly (Bharadwaj et al., 2010).

There are also studies that not only look at protein supplementation alone but also to the combined effect of protein supplementation and exercise like the one from Peak et al. This long (18 months) and large RCT (n=180) with older men (50-79 years) was conducted three years ago. Subjects were randomized into four groups, control, progressive resistance training, calcium – vitamin D3 fortified milk supplement or a combination of the last two. Resistance training consisted of moderate impact weight bearing exercises for three times a week and the supplements were at total of 400 mL. reduced fat milk. Levels of TNF-alpha and IL-6 did not significantly change after 18 months for both the groups with supplementation and with exercise (Peake et al., 2011). In another far smaller study, 36 male and female elderly participated in either a low resistance training program (40% 1-RM) or a high resistance training program (80% 1-RM). Additionally each group ingested a carbohydrate supplement before and during training and a supplement of 22 g. of essential amino acids after exercise. Interventions did not have an effect on TNF-alpha and IL-6 after 12 weeks (Onambélé-Pearson, Breen, & Stewart, 2010b).

In accordance with our research, most published literature does not show significant, positive or negative, effects of protein supplementation on the plasma levels of IL-1beta, IL-6, IL-8 and TNF-Alpha. Only one study did find a reduction of IL-6 and TNF-alpha plasma levels after supplementation with lactoferrin. Due to the limited amount of available literature it is difficult to make a comparison of the results. One of the major differences between the studies is the exact content of the protein supplement itself. Bovine milk and soy milk both contain protein but also many other components. The proteins itself also differ. An effect has been found of lactoferrin while the supplementation of mostly whey and casein proteins, used in this research, do not result in changed plasma cytokine levels.

This current study shows that protein supplementation and resistance-type exercise do not evoke a change in plasma concentrations of IL-1beta, IL-6, IL-8 and TNF-alpha in frail elderly people. Also the combined effect of protein supplementation and resistance type exercise does not give different results, adding strength to the conclusion. With regard to the published literature, which is mostly in accordance, these conclusions form the current consensus.

4.3 High variability in cytokine concentrations

In this study the subjects' mean values per cytokine show a relatively big standard deviation. For IL-6 and -8 the SD is even more than twice the mean value itself. This is displayed in table 3. Although the sample size is relatively large, the extreme values have a big influence on the mean and they also cause large standard deviations. The consequence of these high values is the decrease of statistical significance. It is therefore important to look at the factors that cause this undesirable effect.
Figure 1 shows boxplots of the distribution of the cytokines with ln transformed data for all subjects. It is evident that there are many outliers and even extreme outliers within all groups, even after the most suitable ln transformation of the data. Especially high values can be seen for IL-6 and IL-8, with extreme values up to 39 times as high as the mean value.

Cytokines are proteins secreted by immune mediating cells which are known to undergo up-scaling if needed. Big deviations from baseline are therefore expected in case of acute inflammation. However, the aim of this study is to research low level chronic inflammation. So the question is if the extreme cytokine levels are plausible or the result of some sort of measurement error somewhere along the way. To assess if the extreme values found in this study are "normal" other studies are used as a reference. In a similar study with 138 elderly between 86 and 94 years old a similar range of IL-6 levels was reported. Concentrations ranged between 0.51 and 115.25 pg/mL with an average of 3.2 pg/mL. Although not significant, they found a trend that the frail amongst those elderly had higher IL-6 plasma concentrations (Forsey et al., 2003). In a larger cohort study with 3075 males and females aged between 70 and 79 years old, plasma levels of TNF-alpha of over 16 pg/mL were recorded (Visser et al., 2002). Furthermore, in a more recent cohort study with 55 elderly subjects of mixed gender similar ranges have been reported. Somewhat smaller ranges were found for IL-8 (4.76-217 pg/mL) and IL-6 (0.16-31.5 pg/mL) and wider ranges were found for IL-1beta (0.17-39.0 pg/mL) and TNF-α (0.86-20.8 pg/mL) (Kim, Kim, Youn, Shin, & Park, 2011a).
Compared to other studies with male and female elderly subjects, the highest cytokine levels in this study, although far from the average, seem not to be exceptional. Similar ranges can be found in literature, making it likely that the extreme values are not measurement errors but rather an indication of ongoing or upcoming subclinical inflammation present in some participants.

4.3.1 Alternative analysis

Although the extreme values are plausible they do still have a disproportioned effect on the mean. As mentioned earlier, it has been a topic of discussion in multiple other research studies. The variation in cytokine values between persons might even inhibit a possible effect of the intervention. It is possible that the extreme values represent acute inflammation instead of the chronic inflammation, which was not the desired research subject. Acute inflammation gives rise to higher cytokine concentrations with shorter durations and does not reflect the health associated risks of chronic long term inflammation. To investigate to what extent the extreme values actually affect the outcome, the statistical analysis was conducted again. This time only values within the range of the mean ± 2 standard deviation were included. As a result a total of 20 values were excluded. While a bigger number of outliers in the groups of IL-1beta and TNF-alpha were excluded, mean concentrations were most altered in the IL-6 and IL-8 groups. The mean concentration of IL-6 changed from 2.9 pg/mL to 2.4 pg/mL IL-8 concentration decreased from 12.1 to 9.1 pg/mL Furthermore, as expected, standard deviation and standard error of mean are lowered as a result of the removal of the outliers. This is displayed in Table 4.
The outcome of this study does not change with the removal of the outliers, as can be seen in Table 5. What meets the eye is that the mean IL-8 values in the no exercise – placebo group now resemble those of the other intervention groups. Furthermore, p values of the interventions are even less significant without the extreme values. This shows that the extreme values do not bias an otherwise potential effect of the intervention in this study.

4.4 Mean cytokine values

In the study conducted by Friedenreich et al. no effects of exercise on plasma levels IL-6 and TNF-alpha were found (Friedenreich et al., 2012). The authors suggest that subjects with higher levels of low grade inflammation might experience a different benefit from exercise. In addition Nicklas et al. observed that the reduction of IL-6 plasma levels was driven by the subgroup of subjects who had higher baseline levels of IL-6 (Nicklas et al., 2008). These are indications that studies with higher baseline mean cytokine concentrations tend to be more in favour of a significant outcome than studies with lower mean cytokine levels. It is therefore interesting to investigate how the mean cytokine values of this study compare to that of other studies. After comparison is appears that the baseline mean cytokine concentrations of this study are coherent with most other similar studies. They are in the same order of magnitude. However, a few noticeably different baseline mean values can be found in other studies. Whereas most baseline values are no more than two times bigger than those of this study, some mean baseline values of more than five times as high are reported. The mean baseline concentration of TNF- alpha in the study of Prestes et al. is 72 pg/mL and for Onambélé-Pearson et al. 35. Pg/mL compared to the 4.4 pg/mL of our study, as can be seen in Table 3 (Onambélé-Pearson et al., 2010a; Prestes et al., 2009). Santos et al. even report a mean baseline IL-6 concentration of 151 pg/mL in contrast to the 2.9 pg/mL of this study (Santos et al., 2012). Interesting to see is that, in contrast, they did find a reduction in levels of IL-6 or TNF-alpha after exercise.
That leads to speculation about what can cause these different mean baseline cytokine levels, for the cause could possibly have an effect on the outcome. There are two factors that have a possible influence on the outcome and which keep coming back in discussions. Those are fat mass and state of disease.

4.4.1 Fat mass

It is not that long ago that adipose tissue was seen more as a passive organ of storage than an active regulator of homeostatic systems. Now we know that adipocytes play a role in endocrinology (Wisse et al., 2004). Adipose tissue is recognized as an immune organ that secretes many immunomodulating factors (Kershaw & Flier, 2004; Wisse et al., 2004). Adipocytes can synthesise, among others, IL-6 and TNF-alpha (Coppack et al., 2007) and contribute to about 30% of the total circulating IL-6 concentration (Mohamed-Ali et al., 1997). Therefore systemic IL-6 concentrations increase with an increase in adipose tissue, whereas TNF-alpha does not increase (Coppack et al., 2007; Wisse al., 2004). More evidence for the correlation between fat mass and cytokine levels is delivered by Pedersen et al. They conducted a cross-sectional study with the hypothesis that an altered fat distribution in healthy elderly contributes to high circulating levels of IL-6 and TNF-alpha. Using a bone density scan to measure truncal fat, 20 healthy elderly, 16 elderly with diabetes and 20 young controls were scanned. Results show no difference in cytokine levels between diabetic elderly and healthy elderly subjects. Compared to the young control groups both groups had higher plasma levels. After correction for age and gender the results demonstrated that the relative truncal fat mass was related to high levels IL-6 and TNF-alpha in healthy elderly and elderly patients with type 2 diabetes (Pedersen et al., 2003).

Seeing that fat mass has an influence on systemic IL-6 levels, subjects with more adipose tissue might have higher plasma cytokine levels. This gives reason to believe that lowering body fat mass by means of exercise might have an effect on plasma cytokine levels (Bastard et al., 2000). Practice, however, shows that the correlation between fat mass and cytokine levels does not strongly reflect this. Nicklas et al. found a significant effect of exercise on IL-6 after their 12 month trial. However, no change in body weight or body composition was found. lL-6 was directly related to BMI at baseline, thus indicating that exercise training does not need to result in weight loss to have an effect or at least lowering of IL-6 was not due to lower fat mass. (Nicklas et al., 2008). The authors conclude that exercise either alters IL-6 concentrations with an inhibitory effect on IL-6 production or a stimulatory effect on IL-6 clearance. Similarly, in the study of Onambélé-Pearson et al. body fat did not change. TNF-alpha concentrations did significantly lower, however levels of IL-6 also did not (Onambélé-Pearson et al., 2010a).

In contrast, Wanderley et al. did find a significant reduction in body fat after training but no change in plasma levels of TNF-alpha (Wanderley et al., 2013). Also in a study with obese frail elderly subjects it was found that IL-6 and TNF-alpha decreased significantly by exercise while weight loss had no effect. Although, in contrast to the other studies, cytokine activity was measured on the basis of mRNA activity in upper leg skeletal muscle, instead of blood plasma concentration (Lambert et al., 2008).
In our research BMI was included. With the presumption that a higher BMI indicates more fat mass, BMI would have an effect on the model, this however was not the case. To conclude, the findings of this study and other intervention studies suggest that, regardless of the outcome, exercise induced body fat reduction does not play a big role in the regulation of IL-6 and TNF-alpha plasma levels in elderly.

4.4.2 Disease state

The extend in which people suffer from diseases is different over time and different between persons. People can be knowingly ill but can also suffer unknowingly sub clinically from diseases. State of disease is another common explanation for the possible difference between study outcomes. Instead of measuring levels of chronic inflammation, an acute response or an ongoing chronic disease that all subjects have in common could be measured. In a case control study between elderly and older adults Ok Kim et al. profiled cytokines of a 100 gender matched, healthy subjects. TNF-alpha, IL-6 and IL-1beta levels showed no significant association with age. The author's note that the discrepancy with other studies, which do find an association, might be due to the diseased state of the used subjects, which was not controlled (Kim et al., 2011b). The subjects our study were excluded if they were diagnosed with cancer, had chronic pulmonary diseases or muscle diseases. Furthermore, also subjects with type 2 diabetes (≥ 7 mmol/L) and renal insufficiency (eGFR <60 ml/min/1.73 m^2) were excluded. In the studies that had noticeably higher mean cytokine values compared to this one, all had only included older or elderly subjects without cardiovascular, pulmonary or neurological diseases. Unless the participants of those studies shared another chronic disease it is unlikely that they suffered a known illness that could cause the difference in mean cytokine values. Furthermore, when looking at other researches with participants with a clearly defined illness it can be seen that the baseline mean values do not differ from our study. Patients with coronary heart disease had IL-6 values of 2.50 ± 1.50 pg/mL (Goldhammer et al., 2005). In patients with type II diabetes, values of around 2 and 3 pg/mL for TNF-alpha and IL-6 respectively were found (Giannopoulou et al., 2005). Most exercise intervention studies share the obvious exclusion criteria for diseases that might put a subject at risk, like cardiovascular and pulmonary diseases, this applies also for the studies that have shown higher mean cytokine values. It is possible that subjects those studies suffered from unknown chronic diseases or acute ongoing inflammation. If they all shared the same condition then that would be mentioned. It is well possible that subjects suffered from an acute inflammation, but it is very unlikely that they all did so at once. Therefore the group average wouldn't be affected that much by this. Seeing that those studies had exclusion criteria similar to this current study and that studies which specifically included diseased subject had lower mean cytokine values, the difference in mean cytokine values cannot be contributed to disease state.
However it is still expected that chronic diseases lead to a chronic low grade inflammatory state that is significantly higher than in "healthy" subjects. Apparently this is not the case in all circumstances and not all chronic diseases result in this low grade inflammatory state.

5. Conclusion

Cohort studies show that elevated levels of cytokines are associated with lower muscle mass and muscle strength (Schaap et al., 2006; Visser et al., 2002). Furthermore elderly people with higher activity levels are associated lower levels of inflammation (Addison et al., 2012; Fischer et al., 2007). The ProMuscle study showed a positive effect of resistance type exercise training on strength and physical performance (Tieland et al., 2012a; Tieland et al., 2012b). Our study used the blood samples taken from those subjects, which led to the believe that resistance type exercise training in these same subjects might lead to a decrease in circulating cytokine levels. However, no effects were found of neither resistance-type exercise nor protein supplementation on the plasma concentrations of IL-1beta, IL-6, IL-8 and TNF-alpha in frail elderly people in this 24 week intervention period. The available literature shows this conclusion is shared by most similar intervention studies and is refuted by a few others. Unfortunately our study was troubled by a big inter subject variation which resulted in some extreme outlying values. Even though the outliers in this study are relatively high, after comparison with other similar intervention studies they appeared to be plausible and were therefore not excluded. Even a second statistical analysis without the outliers did not yield a different outcome. This indicates that the outlying values did not impede a possible effect, which makes the conclusion even stronger. In order to make future studies comparable the meaning of the phrasing “exercise” and “protein supplementation” should be very well defined. Training programs and composition of protein supplements should be more alike. Furthermore the health status of the subjects must be well known for disease status has a large influence on blood cytokine concentration and can therefore be a confounder. A large sample size can help counteract this effect.

6. References

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