The role of inflammation in muscle aging

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Cover: *Fluorescent image of muscle cross-section showing a satellite cell at the lower right corner (purple). In Chapter 3 images of this type are used to search for impact of chronic systemic inflammation on muscle aging.*

opgedragen aan mijn oma Elena Manaresi,
door wie mijn affiniteit voor ouderen is ontstaan

Contents

Chapter 1

General Introduction

Muscle aging

Skeletal muscle tissue accounts for 40% by mass for men and 30% by mass for women (Janssen *et al.*, 2000). Age-related decline in muscle mass and strength starts between the age of 30 and 50 years. After the age of 70 years the loss of muscle mass is around 1% per year and the loss of muscle strength around 3% per year (Goodpaster *et al.*, 2006). Low muscle mass and strength is a major contributor to disability and mortality (Filippin *et al.*, 2015).

Sarcopenia

To provide more recognition from the scientific community Rosenberg proposed in 1989 to give age-related low muscle mass and function a name. He suggested to call it *sarcopenia*, derived from the Greek words $\sigma\acute{\alpha}\rho\xi$ = sarx = flesh and $\pi\epsilon\nu\acute{\iota}\alpha$ = penia = poverty, so "poverty of flesh" (Rosenberg, 1989, 1997). Although the number of scientific articles using the term sarcopenia has increased to 750 per year in 2015, its definition is still topic of debate (Reijnierse *et al.*, 2015). There is no consensus on

1. whether the diagnosis also involves low muscle strength (next to low muscle mass) — Clark and Manini (2008) postulated that low muscle strength is a different phenomenon and named it *dynapenia*, Greek for "poverty of strength";

2. whether the diagnosis also involves low physical function (next to low muscle mass and strength) and how this should be measured;

3. which threshold values should be used for the diagnosis;

4. whether the focus should be on muscle mass in the whole body (equivalent to lean body mass) or only on muscle mass in the arms and legs (equivalent to appendicular lean mass);

5. how to adjust muscle mass and strength and physical function for fat mass, and height;

6. which method should be used to measure muscle mass and strength and physical function.

Notwithstanding these uncertainties, in the past decades it has been become more and more clear that the etiology and pathogenesis of age-related low

muscle mass and strength is a complex interplay of a myriad of factors, including physical inactivity, hormonal, metabolic, and nutritional factors (Ali & Garcia, 2014). Research has also implicated inflammation in its pathogenesis (Budui, Rossi & Zamboni, 2015).

Acute and chronic inflammation

Inflammation involves a wide variety of physiological and pathological processes in response to infection, tissue injury, tissue stress (such as hypoxia, which occurs in muscle tissue during exercise), and tissue malfunctioning. One should make a distinction between the *acute inflammatory response* and *chronic inflammation* (Medzhitov, 2008). On the one hand, during the acute inflammatory response, triggered cytokine producing leukocytes such as neutrophils and monocytes from the blood migrate to the site of infection or injury, eliminate the pathogen or injured tissue and stimulate repair (Chazaud, 2014). On the other hand, chronic inflammation leads to tissue damage and the formation of fibrosis (Mann *et al.*, 2011).

Cytokines are involved in the acute inflammatory response as well as in chronic inflammation, but their source differs. Cytokines produced during the acute inflammatory response have as their main source monocytes. Cytokines involved in chronic inflammation have as their main source a wide variety of cell types, including lymphoid cells as well as non-lymphoid cells such as endothelial cells, fibroblasts, and adipocytes (Naka, Nishimoto & Kishimoto, 2002). Moreover, it is known of cytokines like interleukin-6 (IL-6) that they can have pleiotropic effects (Munoz-Canoves *et al.*, 2013). Therefore, it remains an open question whether cytokines like IL-6 have the same effect during the acute inflammatory response as during chronic inflammation, and whether these effects are dependent or independent of each other. We notice in this connection that blood levels of circulating IL-6 are known to be two-fold higher in 90-years old subjects compared to 65-years old subjects, indicating an age-related increase in chronic inflammation (Puzianowska-Kuznicka *et al.*, 2016). However, there are also indications that the acute IL-6 production response decreases over age (Nyugen *et al.*, 2010).

The acute inflammatory response can be estimated using a whole blood stimulation assay. With this assay it is possible to measure the amount of cytokines that are produced within 24 hours in whole blood samples upon stimulation with lipopolysaccharide (LPS), a bacterial component (Damsgaard *et al.*, 2009a). The acute inflammatory response estimated using this method is called immune responsiveness, cytokine production capacity or cytokine production response. Chronic inflammation can be estimated using the data

on circulating levels of cytokines and C-reactive protein (CRP). These levels are referred to as circulating markers of inflammation.

Inflammation and muscle aging

Most research on the relation between inflammation and muscle aging has been focused on the detrimental role of chronic inflammation on muscle mass and strength. For instance, higher levels of circulating IL-6, tumor necrosis factor-alpha (TNF-α) and CRP have been associated with poorer physical performance and a decrease in muscle mass and strength (Ferrucci *et al.*, 2002; Schaap *et al.*, 2009). In mice it has been shown that after acute injury the cytokine producing monocytes infiltrate muscle tissue and are crucial for the stimulation of muscle regeneration (Arnold *et al.*, 2007). This beneficial acute inflammatory response is often overlooked or confused with damaging chronic inflammation. The author of this thesis has encountered this especially in the literature on the effect of physical exercise on chronic inflammation and muscle strength. While several studies show evidence that physical exercise temporarily decreases the acute inflammatory response, for example, Woods *et al.* (2012) instead speak of a decrease in chronic inflammation. This has made the literature on inflammation often confusing and its relation with muscle aging unclear.

Outline of the thesis

The objective of this thesis is to investigate the role of chronic as well as acute inflammation on muscle aging.

In *chapters two and three* we examine the role of chronic inflammation on muscle aging. We studied patients with rheumatoid arthritis (RA), a chronic disease characterized by high levels of circulating inflammatory mediators. RA is used as a disease model for excessive chronic inflammation. If chronic inflammation is one of the main driving factors in muscle aging, we expect to find signs of accelerated muscle aging in RA patients. In *chapter two* we describe the association between muscle strength and age in RA patients and compare it with subjects from the general population. This was investigated using pooled data from 185 studies involving 10149 subjects. In *chapter three* we investigate the age-related histological muscle characteristics in RA patients in comparison to control patients with osteoarthritis (OA). This was investigated using muscle biopsy data from 10 RA and 27 OA patients undergoing elective knee replacement.

In *chapter four* we investigate whether chronic inflammation and the acute inflammatory response are two mutually dependent or independent endo-

types. This was investigated in 403 subjects from the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trail. Firstly, we study the correlation between circulating serum markers of inflammation (as an estimate of chronic inflammation) and cytokine production response measured by a whole blood stimulation assay (as an estimate of acute inflammatory response). Secondly, we study whether the acute inflammatory response has an association with cardiovascular mortality independent of chronic inflammation. Unlike the potential beneficial effects of the acute inflammatory response in skeletal muscle tissue, in the cardiovascular system the acute inflammatory response may have potential damaging effects. This has been suggested by authors reporting a transient increase in risk for a vascular event after infection (Smeeth *et al.*, 2004). If the acute inflammatory response indeed increases the risk for a vascular event, then this should be taken into account in future therapies that increase the acute inflammatory response for the potential benefit of muscle mass and strength.

When the relation between inflammation and diseases is analyzed, the potentially confounding effect of sex differences needs to be addressed. It is known that sex differences in muscle mass and strength are substantial. In order to better interpret the association between the acute inflammatory response and muscle mass and strength, we investigate in *chapter five* the sex differences in the cytokine production response. For this study we used cytokine data from 4020 subjects originating from 15 study populations, either from the general population or from patient populations with specific diseases.

Finally, in *chapter six* we then study the association between cytokine production capacity (as an estimate of acute inflammatory response) and muscle mass and strength in middle-aged elderly. The data from this study originates from 191 men and 195 women from the Leiden Longevity Study, a study consisting of offspring from long-lived Caucasian siblings and the partners thereof.

In *chapter seven* we further explore this association by investigating the relation between interleukin 10 (IL-10) gene variants, known to be associated with cytokine production response (acute inflammatory response) and muscle strength. The research presented in this chapter was performed in 554 elderly from rural Ghana, where muscle strength is of vital importance and pro-inflammatory IL-10 gene variants are enriched. In *chapter eight* the key findings of this thesis are discussed in relation to current literature and recommendations for future research are given.

Chapter 2

Patterns of muscle strength loss with age in the general population and patients with a chronic inflammatory state

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Abstract

Background: There is growing recognition of the serious consequences of sarcopenia on the functionality and autonomy in old age. Recently, the age-related changes in several inflammatory mediators have been implicated in the pathogenesis of sarcopenia. The purposes of this systematic review were two-fold: (1) to describe the patterns of muscle strength loss with age in the general population, and (2) to quantify the loss of muscle strength in rheumatoid arthritis as representative for an underlying inflammatory state. Handgrip strength was used as a proxy for overall muscle strength.
Results: Results from 114 studies (involving 90,520 subjects) and 71 studies (involving 10,529 subjects) were combined in a meta-analysis for the general and rheumatoid arthritis population respectively and standardized at an equal sex distribution. For the general population we showed that between the ages of 25 years and 95 years mean handgrip strength declined from 45.5 kg to 23.2 kg for males and from 27.1 kg to 12.8 kg for females. We noted a steeper handgrip strength decline after 50 years of age (rate of 0.37 kg/year). In the rheumatoid arthritis population handgrip strength was not associated with chronological age between the ages of 35 years and 65 years and was as low as 20.2 kg in male and 15.1 kg in female. Rheumatoid arthritis disease duration was inversely associated with handgrip strength.
Conclusions: This meta-analysis shows distinct patterns of age-related decrease of handgrip strength in the general population. Handgrip strength is strongly associated with the presence and duration of an inflammatory state as rheumatoid arthritis. The putative link between age-related inflammation and sarcopenia mandates further study as it represents a potential target for intervention to maintain functional independence in old age.

2.1 Introduction

Sarcopenia, age-related loss of muscle mass and strength, is highly prevalent, with reported proportions exceeding 50% in those aged 80 years and older (Iannuzzi-Sucich, Prestwood & Kenny, 2002; Baumgartner *et al.*, 1998). This ageing phenomenon is becoming an important public health concern as it inflicts a profound functional burden on our growing elderly population and contributes to increased morbidity (Rantanen, Era & Heikkinen, 1994; Rantanen *et al.*, 1999; Taekema *et al.*, 2010) and mortality (Rantanen *et al.*, 2000; Metter *et al.*, 2002; Ling *et al.*, 2010). Several studies have shown that loss

of muscle mass occurs as early as the fifth decade of life and accelerates in older age (Lexell, Downham & Sjöström, 1986; Janssen *et al.*, 2000). The etiology and pathogenesis of sarcopenia is complex and probably involves the interplay of a myriad of factors including physical inactivity, hormonal, metabolic and nutritional factors (Doherty, 2003; Morley *et al.*, 2001). Recent research has also implicated age-related changes in several inflammatory mediators in the pathogenesis of sarcopenia (Krabbe, Pedersen & Bruunsgaard, 2004).

Similar to the ageing process, inflammatory cytokines have been shown to have a profound role in the pathogenesis of "rheumatoid cachexia", the loss of muscle mass and strength with concomitant increase in fat mass, which persists after joint inflammation improves in rheumatoid arthritis (RA) patients (Roubenoff, 2009). In patients with chronic inflammatory diseases such as RA, there is an accelerated loss of muscle mass and strength compared to healthy subjects (Roubenoff, 2000; Madhok *et al.*, 1993).

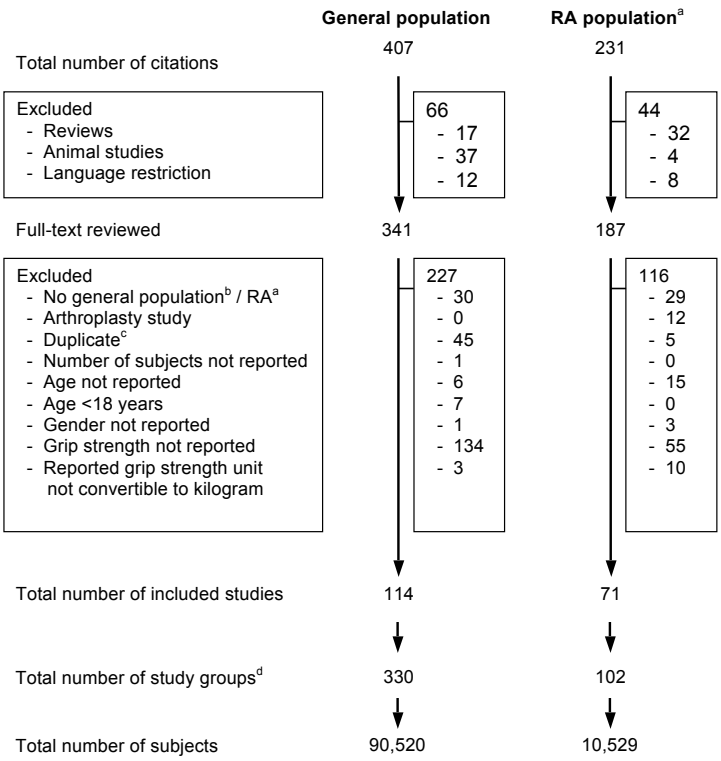
Although sarcopenia research has intensified in recent years, a general overview on the patterns of muscle strength loss with age and chronic inflammation is lacking. The aims of this quantitative systematic review were to describe the patterns of handgrip strength (HGS) loss with age in the general population and to quantify the loss of strength in a chronic inflammatory state such as RA.

2.2 Methods

2.2.1 Selection of studies

We conducted a search of the literature using MESH terms "hand strength", "rheumatoid arthritis" and "ageing" from the MEDLINE database from January 1999 to January 2009. The search was limited to human studies and reports published in English, French, German, Italian or Dutch. Review articles with duplicate data were excluded. The search yielded 528 citations. The article selection process is illustrated in Figure 2.1.

One investigator (K.B.) reviewed all potentially relevant articles in full text. If eligibility was questionable (15% of all articles), articles were discussed with a second investigator (A.M.). Criteria for final inclusion in the systematic review were (1) HGS measurements were performed in subjects representative for the general population or subjects with RA; (2) HGS was measured by a hand-held dynamometer with results reported in kilogram (kg), Newton



[a] RA: rheumatoid arthritis
[b] Studies reporting on admitted subjects or with specific diseases
[c] Studies reporting on a same cohort
[d] Study groups are defined as whole study groups or sub-study groups based on gender, age-categories or study specific sub grouping.

Figure 2.1. Summary of article selection process.

(N) or pound (lb) or a sphygmomanometer with results reported in mmHg or kilo-Pascal and (3) data on sample size, age and gender were available. There were no nationality or ethnicity related selection criteria.

Studies performed exclusively in patients from hospital wards or rehabilitation clinics were excluded as well as those including diseases or conditions that may affect HGS such as osteoarthritis, diabetes, growth hormone or testosterone deficiencies, osteoporosis, severe acute respiratory syndrome, renal failure, neuromuscular disorders (including stroke, dementia and Parkinson's disease), and wrist arthroplasty. Studies with other forms of inflammatory arthritis in the study populations were only included if HGS

measurements were either specifically described for the RA subjects or the RA subjects represented over 75% of the study population.

2.2.2 Data extraction

The following information was extracted from each article: year of publication, sample size, mean and standard deviation (SD) of HGS, age and gender distribution. Whenever available, anthropometrical data, RA disease duration, pain-scores (as measured on the visual analogue scale (VAS)), general health (as measured on the health assessment questionnaire (HAQ)), inflammatory markers (C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR)) were also extracted. When HGS measurements were reported separately for male and female or for different age categories or other study specific sub-grouping, these measurements were regarded as being distinct study groups. Therefore, multiple study groups were extracted from the original articles. In case the age category was reported as range without the mean age, the midpoint of the age range was used for the analysis. Study groups were excluded when no lower or upper age cut-off was described for the age category. For longitudinal studies, only baseline data were selected and HGS measurements reported on subjects who died during the study follow-up duration were excluded.

If necessary, data were converted into kg equivalent: newtons (N) were divided by 9.81; pounds (lb) by 0.45; units of pressure (mmHg) or force (kPa) were converted using validated methods (Desrosiers *et al.*, 1995; Agnew & Maas, 1991). In studies where only the mean HGS was reported without the SD, the latter was estimated based on the linear regression of the variance of HGS on the mean HGS using the groups that provided complete data (mean and SD) for the general or RA population.

2.2.3 Statistical analysis

To determine the mean HGS at different calendar ages in the general and RA population, we performed random effects meta-regression analyses adjusted for age and gender (Van Houwelingen, Arends & Stijnen, 2002). These meta-regression models allowed us to make tables and figures for study groups standardized at 50% females or for males and females separately. For the general population, we first ascertained by eyeballing the scatter plot a change point at which the slope of annual decrease in HGS changed

most rapidly. This change point parameter was then included in the meta-regression analysis as described by Samson *et al.* (2000). To further extend the meta-regression models for both the general and RA population, the method of forward selection was used. We first selected the significant squared terms from the continuous independent variables and then the significant interaction terms. For the meta-regression model of the general population, this resulted in inclusion of the gender x age interaction term in the analysis. This interaction indicated that the relation between HGS and age was dependent on the gender distribution. We plotted this relationship standardized at an equal sex distribution. For the RA population, none of the squared terms or interaction terms were significant and therefore, no further extension of the model was needed. Similarly, the method of forward selection was used in the analysis of HGS and RA disease duration, adjusted for age and gender. This resulted in inclusion of disease duration x age interaction to the meta-regression model. Again, the meta-regression line of this model was only plotted for the case of an equal sex distribution. All statistical analyses were performed in STATA 10. In all meta-analyses the groups were assumed to be independent, i.e. no allowance was made for groups coming from the same study.

2.3 Results

Out of 114 studies related to HGS in the general population we extracted 330 study groups involving 90,520 subjects. For RA, 102 study groups were extracted from 71 studies involving 10,529 subjects (Figure 2.1). In the general population, HGS was measured using a hand-held dynamometer in 99.7% of the study groups and a sphygmomanometer in 0.3%, compared to 61.7% and 38.3%, respectively of the study groups in the RA population. Standard deviation of the mean HGS was reported in 82.1% and 79.4% of the study groups for the general and RA population, respectively.

Table 2.1 shows the characteristics of the extracted study groups for the two populations apart. The median number of subjects per study group was 50.5 in the general population and 47.0 in the RA population. The age range of subjects within all study groups in the general population was 20–100 years (median 65.0) and in the RA population 31–65 years (median 55.1). Clinical parameters (VAS and HAQ scores) and inflammatory markers (CRP and ESR) were reported more frequently in the RA population compared to the general population. The RA population had higher pain score, poorer

Table 2.1. Descriptive statistics of extracted means from study groups included in the review. Study groups derived from 114 studies (330 study groups) of the general population and 71 studies (102 study groups) of the rheumatoid arthritis (RA) population.

	General population		RA population	
	N	median (IQR)	N	median (IQR)
Total number subjects		90,520		10,529
Number of subjects per study group		50.5 (19-189)		47 (20-100)
Age, years	330	65.0 (50.5-75.7)	102	55.1 (50.0-57.5)
Age SD per study group, years	169	5.2 (3.0-7.8)	73	12.0 (10.0-13.0)
Gender, % females	330	54.6 (0-1)	102	77.1 (69.0-87.0)
Disease duration, years	-	-	88	7.8 (2.6-12.5)
Anthropomorphic measurements				
Height, cm	150	163.3 (158.0-172.5)	14	165.5 (164.0-169.0)
Weight, kg	153	66.7 (58.1-76.4)	14	70.8 (67.5-74.0)
BMI, kg/m²	195	25.0 (23.0-26.6)	15	26.0 (25.2-27.4)
Clinical measurements				
Pain, VAS 0-100	3	2 (0-11.5)	51	45.2 (32.0-54.0)
HAQ, 0-3	3	0.05 (0-0.07)	40	1.18 (0.88-1.34)
Inflammatory markers				
CRP, mg/L	8	2.98 (1.0-6.7)	31	22.0 (11.5-32.0)
ESR, mm/h	1	8.8 (-)	51	34.0 (25.0-47.6)

Data are presented as medians with 25th and 75th percentiles (i.e. interquartile ranges [IQR])
N: number of study group with available data. Study groups are defined as whole study groups or sub-study groups based on gender, age categories or study specific sub-grouping.
BMI: body mass index, VAS: visual analogue scale, HAQ: health assessment questionnaire.

general health and elevated inflammatory markers compared to the general population. RA disease duration was reported for 86% of the RA study groups.

Table 2.2 shows the mean HGS values according to age for the general and RA population standardized for males and females separately. In the general population, HGS diminished gradually with age, with a change point at age 50 years after which there was a steeper decline in HGS (Table 2.3 and Figure 2.2). The annual decline of HGS loss was 0.06 kg from age 20 years to 50 years and 0.37 kg from 50 years onwards. In the RA population, HGS values were much lower compared to that of the general population of similar age range. In fact, they were comparable to that of the oldest old in the general population. No association was noted between chronological age and HGS in the RA population (Table 2.3 and Figure 2.3A). However, RA disease duration was significantly associated with a decline in HGS over time, with an annual rate of loss of 0.34 kg at age 55.6 years (95% CI: -0.58 to -0.10) (Figure 2.3B).

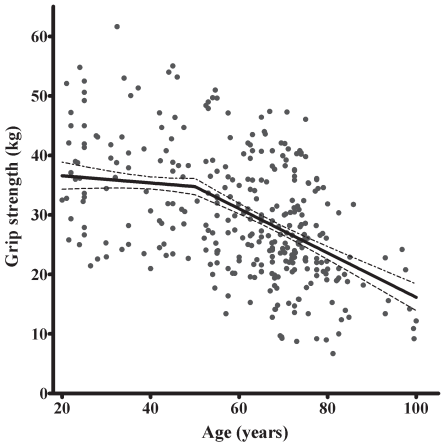


Figure 2.2. Handgrip strength in the general population dependent on age. Scatter plot of study groups from the general population (n = 330) and meta-regression lines with 95% confidence band (standardized at 50% females) with a change point at the age of 50 years.

The availability of additional characteristics, such as anthropomorphic measurements and inflammatory markers was too small to adjust for in the final analysis for the general and RA population. However, when the adjustment was performed on height and weight in the general population, results did not change significantly (data not shown).

2.4 Discussion

We performed meta-regression analyses to evaluate the patterns of HGS loss with age in the general population and quantified the magnitude of strength loss when chronic inflammation is present, using RA as a representative condition. HGS was used as a proxy for overall muscular strength as it has been shown to correlate well with whole body muscle strength [3]. It is also an easily accessible, simple and reliable clinical assessment tool (Innes, 1999). We found an inverse relationship between HGS and age, with a gradual decline starting as early as the third decade of life followed by a steeper deterioration after 50 years of age. Subjects with RA had significantly lower handgrip strength compared to the general population of similar age. HGS was also associated with disease duration of RA. To the best of our knowledge, this

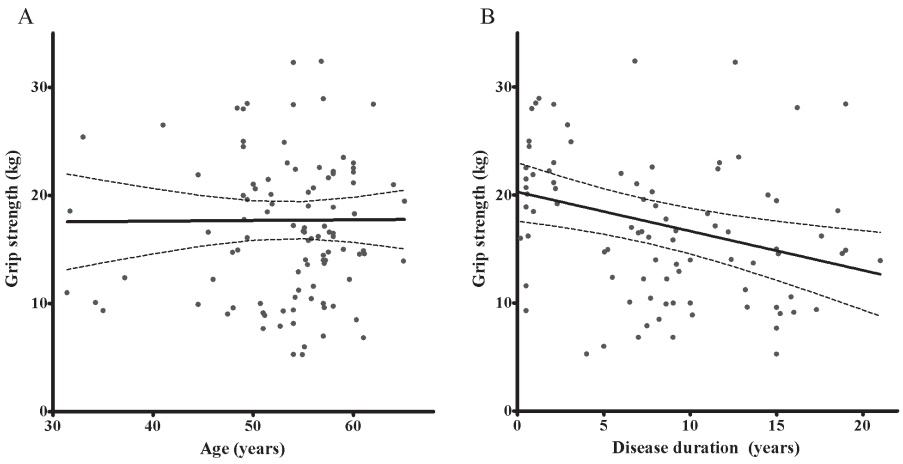


Figure 2.3. Grip strength in rheumatoid arthritis (RA) population dependent on age (n = 102) and RA disease duration (n = 88). (A) Scatter plot of study groups in the RA population dependent on chronological age and meta-regression line with 95% confidence band (standardized at 50% females). (B) Scatter plot of study groups in the RA population dependent on disease duration and meta-regression curve with 95% confidence band standardized at 50% females at median chronological age 55.1 years.

study provides the first large sample systematic review of the patterns of loss of muscle strength in the general as well as the in the RA population over a wide age range.

The data from our study on the pattern of HGS loss in the general population support the curvilinear relationship between HGS and age as reported in some earlier published studies (Vianna, Oliveira & Araújo, 2007; Rantanen *et al.*, 1998). In women, the rapid decline in HGS after 50 years of age has been linked to sex hormone deficiency occurring at menopause and lifestyle changes during the transition (Samson *et al.*, 2000; Vianna, Oliveira & Araújo, 2007). In men however, the profile of strength loss is less well established (Metter *et al.*, 2002; Samson *et al.*, 2000; Vianna, Oliveira & Araújo, 2007). Nonetheless, the rate of HGS decline in the general population of this study comprising of 50% females was comparable to that reported by Rantanen *et al.* (1998).

We also found that HGS in middle-aged RA subjects was approximately half that of the general population. This finding supports previous observations on the negative effects of chronic inflammation on muscle function (Van

Table 2.2. Handgrip strength (kg) in the general population and rheumatoid arthritis (RA) population dependent on age for male and female.

	General population[a]		RA population[b]	
	Male	Female	Male	Female
Age (years)	mean in kg (95% CI)	mean in kg (95% CI)	mean in kg (95% CI)	mean in kg (95% CI)
25	45.5 (43.2; 47.8)	27.1 (24.4; 29.7)	-	-
35	44.3 (42.7; 45.9)	27.0 (25.2; 28.8)	20.2 (15.0; 25.3)	15.0 (11.2; 18.9)
45	43.1 (41.7; 44.6)	27.0 (25.5; 28.5)	20.2 (16.1; 24.4)	15.1 (12.7 ; 17.4)
55	40.4 (39.1; 41.8)	25.4 (24.1; 26.7)	20.3 (16.4; 24.2)	15.1 (13.3; 16.9)
65	36.1 (35.0; 37.2)	22.3 (21.2; 23.3)	20.4 (16.0; 24.7)	15.2 (12.4; 18.0)
75	31.8 (30.5; 33.2)	19.1 (17.9; 20.3)	-	-
85	27.5 (25.6; 29.5)	16.0 (14.2; 17.7)	-	-
95	23.2 (20.6; 25.8)	12.8 (10.4; 15.2)	-	-

Grip strength in kg of each age is standardized for male and female.
[a] Meta-regression model with change point at age 50 years.
[b] Linear meta-regression model.
CI: confidence interval.

Table 2.3. Change in handgrip strength (kg) per calendar year in the general population and rheumatoid arthritis (RA) population.

	General population[a]		RA population[b]	
	Age range in years	mean (95% CI)	Age range in years	mean (95% CI)
Change of grip strength (kg/y)	20-50	-0.06 (-0.16; 0.04)	31-65	0.01 (-0.18; 0.19)
	50-100	-0.37 (-0.44; -0.31)		

Standardized at 50% females.
[a] Meta-regression model with change point at age 50 years.
[b] Linear meta-regression model.
CI: confidence interval.

Hall *et al.*, 2008; Bodell *et al.*, 2009). Few studies have reported the crucial role of "sarcoactive" cytokines such as TNF-α and IL-6 and CRP in the pathogenesis of RA (Madhok *et al.*, 1993; Choy & Panayi, 2001; Engvall *et al.*, 2008). These pro-inflammatory cytokines have also been implicated in the pathogenesis of sarcopenia in "normal" ageing (Schaap *et al.*, 2009).

In RA subjects loss of muscle strength was already observed before the age of 50 years. This supports the concept of RA being a disease of accelerated aging. Recenty it has been shown that RA patients suffer from excess ageing occurring prior to RA incidence as well as an acceleration of aging (Schaap *et al.*, 2009). We postulate that the loss of muscle strength early in life of RA subjects is one of the aspects behind this phenomenon.

Our quantitative analysis of the literature has several limitations. First, there is the limitation due to using aggregate data (study groups) instead of data of individuals. However, the variance that is lost by aggregating individu-

als into study groups in the general population is limited, because the median SD of age for the study groups is calculated to be only 5.2 years, which is small compared to the between studies variance in mean age. In RA the median SD of age was higher (12.0 years) which could have led to an underestimation of the effect of age on HGS. Despite of aggregating, the statistical heterogeneity between study groups was high. This might be caused by differences between studies in factors such as method of HGS measurement, body composition and ethnicity and in the RA population also by RA disease duration and medication use.

We were unable to directly assess the influence of inflammation on HGS in the RA subjects as we did not account for various other factors that could affect HGS measurements such as pain or fear of pain, stiffness, corticosteroid use, disuse atrophy and mechanical disruption. Furthermore, in the RA population, we observed an inverse association between HGS and duration of RA disease but not the influence of chronological age per se. This could in part be explained by selection as RA subjects with profound muscle weakness were less likely to participate in studies and had higher mortality due systemic disease. Still, the amount of inflammatory pressure during the ageing process is low when compared to RA (Madhok *et al.*, 1993). We showed that at the moment of RA diagnosis mean HGS is already as low as 20 kg. This implies that the impact of chronic inflammation on HGS was the highest already during the months from the earliest onset of RA and at the moment of diagnosis, which normally goes together with the start of an anti-inflammatory treatment (Morel & Combe, 2005). For the period after diagnosis we showed RA to be associated with remarkable elevated level of inflammatory markers ESR and CRP.

This meta-analysis confirms previously reported patterns of age-related decrease of HGS in the general population. The putative link between age-related inflammation and sarcopenia mandates further exploration. Investigating RA may serve as representative condition when studying inflammatory pathways that lead to sarcopenia, which are relatively subtle in the general population. These pathways, however represent a potential target for intervention to prevent disability and maintain functional independence in old age.

Chapter 3

Muscle characteristics in patients with an underlying inflammatory state

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Abstract

Background: Histological characteristics of age-related muscle wasting are type II muscle fiber atrophy, accumulation of oxidative stress-induced lipofuscin granules and decreased satellite cell numbers. There is increasing clinical evidence for a strong correlation between chronic systemic inflammation and age-related muscle wasting. The aim of this study was to determine the impact of chronic systemic inflammation on age-related histological muscle characteristics.
Methods: As a model for chronic systemic inflammation, we included 10 patients suffering from rheumatoid arthritis (RA) and 27 control patients suffering from osteoarthritis (OA). Biopsies were taken from the vastus medialis muscle.
Results: No significant differences were found in type II muscle fiber atrophy, lipofuscin accumulation, or satellite cell number in RA compared to OA patients.
Conclusions: These results suggest there is no association between chronic systemic inflammation in RA and age-related muscle characteristics. Future research should focus on inflammation and satellite cell function.

3.1 Introduction

The age-related loss of skeletal muscle mass (sarcopenia) is a major contributor to disability and mortality (Hairi *et al.*, 2010; Szulc *et al.*, 2010; Ling *et al.*, 2010). Between the ages of 20 and 80 years the average reduction in muscle cross-sectional area amounts to 40% (Lexell *et al.*, 1988). The size and numbers of muscle fibers are under control of satellite cells, the muscle progenitor cells that lie inactivated between the basal lamina and the sarcolemma (Mauro, 1961). A decline in satellite cell number, together with type II muscle fiber atrophy has been reported to occur during aging (Verdijk *et al.*, 2007; Kadi *et al.*, 2004). Morphologically, muscle fibers and satellite cells of elderly subjects show an accumulation of lipofuscin granules, a marker for oxidative damage (Roth *et al.*, 2000; Hutter *et al.*, 2007). Despite its clinical importance, the pathophysiological mechanisms behind the development of sarcopenia are not yet well known.

A possible cause for sarcopenia is systemic, low grade chronic inflammation. Increased systemic pro-inflammatory cytokine levels have been observed during ageing (Krabbe, Pedersen & Bruunsgaard, 2004), and they

have been associated recently with poor muscle strength independent of diseases, smoking or physical exercise (Tiainen *et al.*, 2010). In patients suffering from rheumatoid arthritis (RA) the levels of inflammatory markers are high already at middle age, despite anti-inflammatory treatment (Zangerle *et al.*, 1992; Flytlie *et al.*, 2010). In these patients, muscle strength is significantly lower compared to the general population (Slatkowsky-Christensen *et al.*, 2007; Beenakker *et al.*, 2010).

In this study we aimed to determine the impact of chronic systemic inflammation on age-related histological muscle characteristics, type II muscle fiber atrophy, the level of lipofuscin accumulation and satellite cell number per fiber. As a model for chronic systemic inflammation, we examined muscle biopsies from patients with RA who have a significantly higher pro-inflammatory profile when compared to patients with osteoarthritis (OA) (Zangerle *et al.*, 1992; Kimura *et al.*, 2007).

3.2 Material and Methods

3.2.1 Study population

The study population included patients suffering from RA (n = 10) and OA as controls (n = 27) who underwent elective knee replacement surgery in the period 2008 to 2010. Patients with knee revision surgery, tumor surgery, acute trauma, osteonecrosis, myositis or ankylosing spondylitis were excluded. RA patients were matched with OA patients on gender and age. Height and weight were assessed preoperatively, and inactivity (0 to 10) and pain scores (0 to 10) were assessed using the Dutch version of the AIMS questionnaire (Riemsma *et al.*, 1996). Preoperative blood samples were taken for measurement of C-reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR) and white blood cell (WBC) concentration. Anti-inflammatory medication use, including prednisone, methotrexate and TNF inhibitors during the 5 years prior to surgery and the presence of peripheral neuropathy and lumbar discopathy were collected from medical charts. The study protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center.

3.2.2 Muscle biopsies

A muscle biopsy was taken from the distal part of the vastus medialis muscle during elective knee replacement surgery. After embedding in Tissue-Tek

(Sakura Finetek, the Netherlands) biopsies were frozen in liquid nitrogen and stored at −80˚C until further analysis. Muscle biopsies were cut in sections of 10 μm and 5 μm at −21˚C using a cryostat-microtome (Leica Instruments GmbH, Nussloch, Germany) and mounted on uncoated microscope slides (Starfrost, Braunschweig, Germany). Immediately after cutting, the sections were evaluated for cross-sectional alignment. Biopsies from RA and OA patients were stained simultaneously and blinded for patient characteristics.

3.2.3 Fiber type staining

To determine muscle fiber type, 10 μm sections were stored at −20˚C for a maximum of one night. The non-reversed method for ATPase staining was used as described by Round, Matthews & Jones (1980). Briefly, slides were incubated for 30 minutes in 10 mg ATP (ATP disodium salt, Sigma Chemicals Ltd) and dithiothreitol in buffered calcium chloride at pH 9.4 at 37˚C. Second, slides were washed in calcium chloride 1% and incubated in cobalt chloride 2% for two minutes. Afterwards, slides were washed very thoroughly in distilled water and incubated in an ammonium sulphide 1% solution for 30 seconds.

3.2.4 Lipofuscin staining

Lipofuscin accumulation was determined using two 5 μm sections and the level of lipofuscin specific auto-fluorescence was determined (Hutter *et al.*, 2007). To localize the cross-sectional muscle fibers, the fiber membrane was stained with PC128 (dilution 1:500, sheep anti-laminin, The Binding Site, Birmingham, U.K) as primary antibody and Cy5 (dilution 1:50) as secondary antibody. Stained sections were stored at 4˚C until images were captured.

3.2.5 Satellite cell staining

Satellite cells were stained as described earlier by Lindstrom & Thornell (2009). Two 5 μm sections were fixed in 2% formaldehyde for 8 minutes and rinsed in 0.01M phosphate buffered saline containing 0.05% Tween 20 (VWR Prolabo, Fontenay-sous-bois, France) 3 times for 5 minutes each. Sections were blocked with IgG-free bovine serum antigen 4% (Jackson Immuno Research, West Grove, Pennsylvania) for 90 minutes (first primary anti-body incubation) and for 60 minutes (second primary antibody incubation). First, sections were incubated with CD56 (dilution 1:3, mouse anti-

CD56; BD biosciences, San Jose, CA) overnight at 4˚C followed by incubation with FITC (dilution 1:50). Second, sections were incubated with Pax7 (dilution of 1:10, Mouse anti-PAX-7, Developmental Studies Hybridoma Bank, Iowa City, U.S.A.) and PC128 (dilution 1:500, Sheep anti-laminin, The Binding Site, Birmingham, U.K.) for 60 minutes at room temperature. PC128 were labeled with Cy5 (dilution 1:50) and Pax7 with Rhodamine Red (dilution 1:200). All secondary antibodies were purchased from Jackson Immuno Research, West Grove, Pennsylvania. Nuclei were stained with Hoechst 33258 (Molecular Probes, Leiden, The Netherlands) for 5 minutes at room temperature. Sections were washed with PBS 3 times for 5 minutes each, mounted with aqueous mounting medium (Dako, Carpinteria, U.S.A.) and covered with cover glasses. Slides were stored at 4˚C for a maximum of 3 days until images were captured.

3.2.6 Image capture and analysis

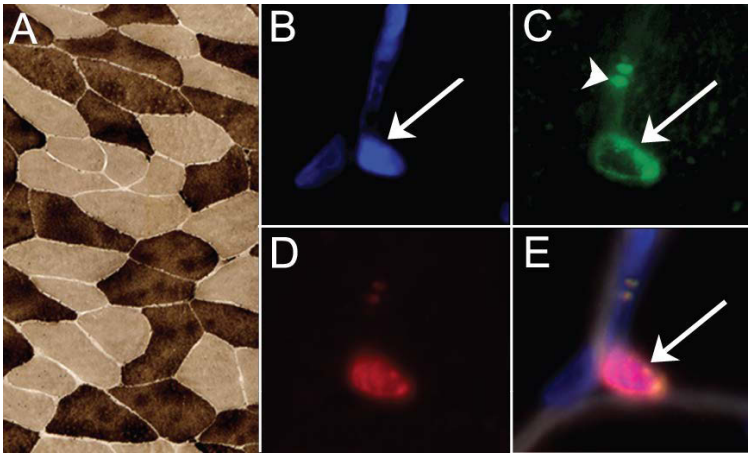


Figure 3.1. Images of muscle cross-sections stained for muscle fiber typing and satellite cell identification by multi-labeling. A: Staining of type I (white) and type II (black) muscle fibers B: DAPI staining (blue), C: N-CAM staining (green), D: Pax7 staining (red), E: Combination of DAPI, N-CAM, Pax7 and laminin (white). The satellite cell is indicated by an arrow. Lipofuscin granules are indicated by an arrowhead.

All images were captured and analyzed blinded for patient characteristics. Slides stained for muscle fiber type determination were scanned using a 3Dhistech automatic digital slide scanner (Panoramic Midi, 3Dhistech) with a 20x magnification (Figure 3.1). For each patient a minimum of 200 (mean

339 for type I fibers and 596 for type II fibers) muscle fibers were randomly selected. The cross-sectional area of muscle fibers was measured using the program HistoQuant (3Dhistech). Fibers were counted using Image-J software, version 1.43. We quantified type II fiber atrophy by 1) the ratio of the mean area of single type II fibers to that of single type I fibers and 2) the number of type II fibers related to the total number of fibers. However, in one RA and three OA patients fiber type measurement was not possible due to poor staining quality or low number of muscle fibers.

Two hundred cross-sectional muscle fibers were captured at 40x magnification to quantify lipofuscin accumulation using a conventional Leica DM 5500 B fluorescence microscope (Leica, Rijswijk, The Netherlands) together with a CoolSnap K4 cooled charge-coupled device camera (Roper Scientific, Evry Cedex, France). Contrast was enhanced, and the total gray value was measured using Image-J software version 1.43.

Satellite cells were visualized using the same fluorescence microscope and camera as was used for the lipofuscin measurements. At 40x magnification images were captured and analyzed for at least 200 cross-sectional muscle fibers per patient. For this procedure the software program Colorproc was used to automatically decrease background staining. Satellite cell number was measured in 5 RA and 12 OA patients due to limited biomaterial. Cells were identified as satellite cells if they were stained by Pax7 or NCAM containing a nucleus stained by Hoechst and resided in a sublaminar position confirmed by the laminin staining (Figure 3.1). Satellite cells were identified and counted blinded to group by two independent observers. Disagreement was resolved by consensus. Satellite cell number was determined in relation to the number of muscle fibers within an image using the formula: number of counted satellite cells / number of counted muscle fibers X 100.

3.2.7 Statistical analysis

The independent-samples t-Test was used to test for differences in age, height, weight, inactivity score, pain score, CRP, ESR and WBC concentration between RA and OA patients. Differences in distribution of gender, the use of prednisone, methotrexate and TNF inhibitors and peripheral neuropathy and lumbar discopathy were tested by use of the Mann-Whitney test. Linear regression models were used to test for differences between RA and OA patients and for associations between muscle characteristics and CRP, ESR and WBC concentration. All models were adjusted for age, gender, height and weight. Analyses were performed using SPSS software (version

16.0 SPSS Inc, Chicago, USA). All P values below 0.05 were considered to be statistically significant.

3.3 Results

The ratio of single type II and type I fiber areas, the percentage of type II fibers II, the level of lipofuscin accumulation and the number of satellite cells per fiber were compared between patients with RA and OA. Characteristics of the test subjects are given in Table 3.1. The mean age of the RA group was 63.6 years (SD 9.1), and it was 66.0 years (SD 8.4) in the OA group. The gender distribution and pain scores were comparable between the groups. The inactivity score was not significantly higher in the RA group compared to the OA group. The mean duration of RA disease was 22.6 years (SD 13.0). All markers of inflammation as well as anti-inflammatory medication use were significantly higher in RA patients compared to OA patients.

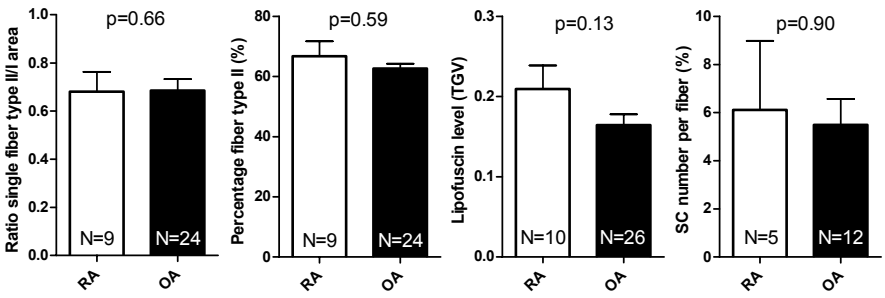


Figure 3.2. Ratio of type II and type I single fiber areas, percentage type II fibers, lipofuscin level and satellite cell number per fiber in muscle tissue from RA and OA patients. Results are given as mean and standard error. P-values are calculated using linear regression analysis adjusted for age, gender, height and weight. RA: rheumatoid arthritis OA: osteoarthritis, Ratio of single type II/I area: ratio of the area of single type II fibers to that of single type I fibers. Percentage type II fibers: number of type II fibers related to the total number of fibers. TGV: total gray value, SC: satellite cell.

Figure 3.2 shows the histological characteristics of the muscle tissue in patients with RA and OA. The mean ratio of type II and type I single fiber areas was 0.68 (SE 0.25) in the RA group versus 0.69 (SE 0.23) in the OA

group. The mean percentage type II fibers was 66.8% (SE 4.9) in the RA group and 62.7% (SE 1.6) in the OA group. The mean lipofuscin value was 0.21 (SE 0.03) in the RA group and 0.16 (SE 0.01) in the OA group. The number of satellite cells per fiber was 6.1% (SE 2.9) in RA patients and 5.5% (SE 1.1) in OA patients. After adjustment for gender, age, height and weight no significant differences in the ratio of type II and type I single fiber areas, the percentage of type II fibers, the level of lipofuscin accumulation and the number of satellite cells per fiber were found between RA and OA patients. Independent of RA and OA diagnosis, no significant association was found between markers of inflammation and histological muscle characteristics (Table 3.2).

3.4 Discussion

Chronic systemic inflammation is suggested to play an important role in muscle wasting during aging (Beenakker *et al.*, 2010; Schaap *et al.*, 2009; Wiroth *et al.*, 2005). To test for the impact of chronic systemic inflammation on characteristics associated with muscle aging, we compared muscle biopsies obtained from patients with an inflammatory disease (RA) and control patients (OA). No significant differences were found in type II muscle fiber atrophy, the level of lipofuscin accumulation or the number of satellite cells per fiber in patients with RA compared to OA patients.

Multiple factors, including age and inactivity, are known to cause type II muscle fiber atrophy (Lexell *et al.*, 1988; Banker & Engel, 2004). Both RA and OA are associated with a lower activity level (Arne *et al.*, 2009; Safdar *et al.*, 2010) and type II muscle fiber atrophy (Edstrom & Nordemar, 1974; Fiori *et al.*, 1983; Magyar *et al.*, 1977; Nakamura & Suzuki, 1992; Reardon *et al.*, 2001; Fink *et al.*, 2007). Although the RA group had a slightly higher inactivity score compared to the OA group, neither the ratio of type II and type I single fiber areas nor the percentage of type II single fibers differed between groups. An earlier study compared muscle biopsies from RA and OA patients and found a significantly higher cross-sectional area of type II muscle fibers, but no significant difference in the ratio of type II and type I single fiber areas in 29 RA biopsies compared to 16 OA biopsies. The limitation of that study was that inactivity and pain levels were not measured (Touno *et al.*, 1996). We conclude that chronic systemic inflammation in RA has no additional contribution to type II muscle fiber type II.

Lipofuscin accumulation levels are a robust marker for past oxidative

stress, which contributes to decline in muscle function in older adults (Hutter *et al.*, 2007; Howard *et al.*, 2007). Reactive oxygen species have also been described to play a role in the pathophysiology of RA (Biemond, Swaak & Koster, 1984; Sarban *et al.*, 2005). A lower antioxidant enzyme activity and a higher level of oxidative damage products were observed in erythrocytes from RA patients compared to OA patients (Sarban *et al.*, 2005). Compared to healthy controls, RA patients have a higher number of lipofuscin granules in the vastus lateralis muscle, when investigated by electron microscopy (Wroblewski & Nordemar, 1975). We found slightly higher levels of lipofuscin accumulation in patients with RA compared to patients with OA, but the difference was not statistically significant. It is possible that this difference would reach significance if more RA patients were included. Interestingly, physical inactivity due to OA has recently been associated with reduced mitochondrial function within skeletal muscle cells and with chronic inflammation (Safdar *et al.*, 2010). A contribution of sedentary lifestyle to the accumulation of cellular damage cannot be excluded.

During chronological aging the number of satellite cells is known to decline (Kadi & Ponsot, 2010). Rodent studies have shown that high concentrations of inflammatory cytokines stimulate apoptosis of satellite cells in vitro (Degens, 2010). A comparable study analyzing human satellite cell characteristics found that inflammatory cytokines stimulate the proliferation of satellite cells and inhibit their initiation of differentiation, but they do not induce apoptosis (Foulstone *et al.*, 2004). In vivo, it has been show that gene transfer of the pro-inflammatory factor TNF-α in mice causes a significant reduction in number and size of regenerating fibers following muscle injury (Coletti *et al.*, 2005). Because RA patients are known to have higher concentrations of inflammatory markers compared to OA we hypothesized that this would lead to a reduced regenerative potential in RA, including a lower number of satellite cells. This is the first time that the number of satellite cells per fiber is quantified in patients with RA. Two studies examined RA muscle biopsies by electron microscopy. Using this method it is not possible to quantify satellite cells in relation to the number of fibers (Thornell *et al.*, 2003). No satellite cells at all were found in intrafusal muscle fibers around muscle spindles in 100 RA patients (Magyar *et al.*, 1979). Another study found a higher number of satellite cells in muscle biopsies from twelve RA patients compared to healthy controls (Wroblewski & Nordemar, 1975). We reported no difference in the number of satellite cells per fiber in RA and OA patients, indicating that a link between chronic systemic inflammation in RA and satellite cell number

is unlikely.

Recently, it has been shown that higher CRP levels and a higher ESR in RA patients are associated with a lower lean body mass and the presence of sarcopenic obesity (Dao, Do & Sakamoto, 2011; Engvall *et al.*, 2008). In this study we found that type II fiber atrophy, lipofuscin accumulation, and satellite cell number per fiber were not significantly associated with ESR, CRP or WBC concentration. Furthermore, it is known that several inflammatory cytokines are capable of accelerating muscle proteolysis (Mitch & Goldberg, 1996). However, the precise mechanism by which this would cause muscle wasting in RA or during the aging process has yet to be elucidated (Walsmith & Roubenoff, 2002).

The strength of this study is the long disease duration and high levels of inflammation in RA patients compared to controls. Furthermore, both groups showed the same age and gender distribution. A limitation of this study is the relatively low number of patients with RA included in the satellite cell measurements. The effect of anti-inflammatory medication use like prednisone, methotrexate and TNF inhibitors could also have diluted the studied effect. In addition, OA patients are known to have higher levels of systemic inflammatory markers compared to healthy controls (Mishra *et al.*, 2011). This low-grade inflammation in OA may have caused a substantial detrimental effect on skeletal muscle. Using OA as a control group for RA patients would therefore underestimate the impact of high-grade systemic inflammation on age-related muscle characteristics.

In conclusion, chronic systemic inflammation in RA is unlikely to be associated with type II muscle fiber atrophy, lipofuscin accumulation, or number of satellite cells per fiber. Further investigations should focus on chronic inflammation and satellite cell function such as activation and proliferation.

Table 3.1. Characteristics of study subjects.

Characteristic	RA-group n=10	OA-group n=27	P
Age, years	63.6 (9.05)	66.0 (8.41)	NS
Gender, % female	80	81	NS
Disease duration, years	22.6 (13.0)	n/a	n/a
Height, cm	169.9 (11.7)	165.3 (6.7)	NS
Weight, kg	85.0 (19.9)	86.4 (17.3)	NS
Clinical measurements*			
Inactivity score, 0-10	3.9 (2.9)	2.5 (2.0)	NS
Pain score, 0-10	5.4 (2.6)	6.6 (2.1)	NS
Markers of inflammation			
CRP, mg/l	11.8 (10.8)	3.3 (4.9)†	0.038
ESR, mm	23.8 (14.9)	10.2 (6.3)‡	0.020
WBC, $*10^9$/L	9.4 (2.8)	7.2 (1.5)§	0.042
Anti-inflammatory medication			
Prednisone, n (%)	3 (30)	1 (4)	0.024
Methotrexate, n (%)	8 (80)	0	<0.001
TNF inhibitors, n (%)	3 (30)	0	0.003
Neurologic diseases			
Peripheral neuropathy, n (%)	0	2 (7)	NS
Lumbar discopathy, n (%)	1 (10)	1 (4)	NS

RA: rheumatoid arthritis, OA: osteoarthritis, CRP: C-reactive protein, ESR: Erythrocyte sedimentation rate, WBC: White blood cells, TNF: Tumor necrosis factor, P: P-value, NS: not significant. Values are given as mean (SD) if not otherwise stated. Dutch version of the AIMS questionnaire was used to assess the inactivity and pain scores. *available for 4 RA and 16 OA patients. †available for 18 OA patients. ‡available for 11 OA patients. §available for 22 OA patients.

Table 3.2. Association between inflammatory markers and muscle characteristics.

Inflammatory marker	n	Ratio single fiber type II/I area		Percentage fiber type II (%)		Lipofuscin (TGV)*		SC number per fiber (%)†	
		β (SE)	P	β (SE)	P	β (SE)	P	β (SE)	P
CRP, mg/l	25	0.008 (0.006)	0.21	-0.35 (0.28)	0.23	-0.001 (0.002)	0.57	0.1 (0.3)	0.59
ESR, mm	19	-0.002 (0.006)	0.72	0.25 (0.22)	0.28	0.001 (0.002)	0.63	-0.3 (0.3)	0.50
WBC, $*10^9$/L	28	-0.004 (0.025)	0.86	1.05 (1.16)	0.37	-0.004 (0.009)	0.67	-0.7 (0.6)	0.27

Adjusted for age, gender, height, weight and rheumatoid arthritis and osteoarthritis diagnosis by linear regression. Ratio of single type II/I area: ratio of the area of a single type II fibers to that of single type I fibers. Percentage type II fibers: number of type II fibers related to the total number of fibers. TGV: Total gray value, SC: Satellite cell, n: number of patients, β: beta, SE: standard error, P: P-value, CRP: C-reactive protein, ESR: Erythrocyte sedimentation rate, WBC: White blood cells. * n available for CRP: 28, ESR: 21, WBC: 32. † n available for CRP: 13, ESR: 9, WBC: 13.

Chapter 4

Immune responsiveness associates with cardiovascular mortality independent of circulating markers of inflammation

Submitted for publication by: K.G.M. Beenakker, S. Chen, M.P. Rozing, members of the PROSPER-group, R.G.J. Westendorp, A.B. Maier

Abstract

Background: Recent studies showed that the risk of a cardiovascular event is transiently increased after infection. This suggests a possible role for acute elevation of cytokines during an immune response in the development of cardiovascular disease. The aim of this study is to investigate whether immune responsiveness associates with cardiovascular, non-cardiovascular and all-cause mortality and whether this association is dependent on circulating markers of inflammation.
Methods: In 403 subjects from the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trail, with a mean age of 75.1 years, we determined immune responsiveness at baseline by ex-vivo stimulating whole-blood samples with lipopolysaccharide (LPS) and measuring the interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-12, IL-1β, IL-10, and IL-1RA production within 24 hours. An immune responsiveness composite score was obtained by averaging the individual cytokines' sex-specific standardized production responsiveness values. Serum IL-6 and high-sensitivity C-reactive protein (Hs-CRP) were measured as circulating markers of inflammation. Subjects were followed for 12.8 years and cardiovascular, non-cardiovascular and all-cause mortality was recorded.
Results: A higher IL-6, TNF-α, and IL-1β production responsiveness was associated with a significantly higher cardiovascular and all-cause mortality. The hazard ratio (95% confidence interval; P-value) per standard deviation increment in immune responsiveness composite score was 1.89 (1.26–2.85; $P = 0.002$) for cardiovascular mortality and 1.39 (1.12–1.74; $P = 0.003$) for all-cause mortality. Adjusting these relations for circulating markers of inflammation did not change the results.
Conclusion: Immune responsiveness associates positively with the risk of cardiovascular and all-cause mortality independent of circulating markers of inflammation.

4.1 Introduction

Inflammatory processes are considered major contributors to the development of cardiovascular disease. These processes are regulated by cytokines that interact with endothelial cells, vascular smooth muscle cells, and extracellular matrix and that are associated with vascular dysfunction and atherosclerosis (Sprague & Khalil, 2009). Recent genetic studies showed that the

receptor of interleukin-6 (IL-6) plays a causal role in coronary heart disease (Collaboration IRGCERF, 2012; IL6RMR Consortium, 2012). Chronically elevated levels of circulating cytokines like serum IL-6 and other circulating markers of inflammation such as high-sensitivity C-reactive protein (Hs-CRP) are well-known risk factors for cardiovascular disease (Singh & Newman, 2011). Moreover, it has also been shown that there is a transient increase in the risk of a vascular event after infection (Warren-Gash, Smeeth & Hayward, 2009). It has therefore been suggested that acute elevations of cytokines levels are also involved in the development of cardiovascular disease (Smeeth *et al.*, 2004).

An *ex-vivo* whole-blood stimulation assay has been developed to investigate immune responsiveness (Desch *et al.*, 1989). This stimulation assay is well reproducible and assesses primarily subject's monocytic cytokine production response upon stimulation with lipopolysaccharide (LPS) (Damsgaard *et al.*, 2009a), which is under thigh genetic control (De Craen *et al.*, 2005). Immune responsiveness measured by this method has been associated with cardiovascular mortality in women aged 85 year old (Van den Biggelaar *et al.*, 2004). However, it is currently unknown whether immune responsiveness also relates to cardiovascular mortality in men and younger individuals and whether elevated levels of circulating markers of inflammation play a role in this association.

The aim of the present study was to investigate whether immune responsiveness, determined by measuring whole-blood's cytokine production responsiveness upon stimulation with LPS, relates to cardiovascular, non-cardiovascular and all-cause mortality. Furthermore, we assessed whether this relation is dependent on circulating markers of inflammation.

4.2 Material and Methods

4.2.1 Study design and subjects

Within the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER), a large multicenter randomized placebo-controlled trial including 5804 subjects, it was assessed whether treatment with pravastatin decreases the risk of major vascular events in elderly. A detailed description of the protocol has been published elsewhere (Shepherd *et al.*, 1999, 2002). Between December 1997 and May 1999 subjects were screened and enrolled in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Subjects were

men or women aged 70-82 years with either preexisting vascular disease or increased risk of vascular disease because of smoking, hypertension, or diabetes mellitus. Subjects with congestive heart failure, arrhythmia or a history of malignancy within 5 years prior to the trial were not eligible to participate. In a substudy, including a random sample of 403 subjects (30%) of the subjects in the Netherlands, immune responsiveness was measured at baseline.

4.2.2 Laboratory measurements

Immune responsiveness was assessed by measuring the level of IL-6, tumor necrosis factor (TNF)-α, IL-12, IL-1β, IL-10, and IL-1RA produced by whole-blood samples upon ex-vivo stimulation with 10 ng/ml LPS during 24 hours at standard culture condition (37˚C, 5% CO_2), as described earlier (Van der Linden *et al.*, 1998). Cytokine levels outside the range of three standard deviations were regarded as outliers and excluded from the analyses (0.7% of the cytokine values). Hs-CRP was measured on stored (at −80˚C) and previously unthawed samples by automated particle-enhanced immunoturbidimetric assay (Roche UK, Welwyn Garden City, UK). Serum IL-6 was assayed using a high-sensitivity ELISA (R & D Systems, Oxford, UK).

4.2.3 Other baseline measurements

A research nurse interviewed all subjects to obtain data on demographic characteristics. Body mass index was calculated using standard protocols. Each participant's general practitioner provided information about history of vascular diseases (coronary, cerebral, or peripheral). Diabetes mellitus was defined by self-reported history, a fasting glucose concentration of 7.0 mmol/L, or self-reported use of anti-diabetic medication.

4.2.4 Follow-up measurements

Subjects were followed for mortality until January 1, 2012 in an average follow-up period of 12.8 years. Dates of deaths were obtained from the Dutch civil registry and specific data on causes of death from the Dutch Central Bureau of Statistics. Death due to cardiovascular mortality was classified as ICD-10 codes 100–199 and death due to other reasons was classified as non-cardiovascular mortality.

4.2.5 Statistical analyses

Immune responsiveness values as well as circulating markers of inflammation values were natural log-transformed due to skewness. For each of the cytokines a sex-specific Z-score was calculated to be able to compare effect sizes and to combine them in composite scores. The immune responsiveness composite score was obtained by averaging the IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA sex-specific Z-scores. Data on one (n = 21), two (n = 1) or three (n = 3) of these cytokines were not available. In case of missing data, the composite score was obtained by averaging the sex-specific Z-scores of the remaining cytokines. The circulating markers of inflammation composite score was obtained by averaging the sex-specific Z-scores for serum IL-6 and Hs-CRP. If data on one of these circulating markers was not available (n = 1), then the sex-specific Z-scores of the remaining marker was used. A partial correlation between immune responsiveness values and circulating markers of inflammation values was calculated adjusted for age, smoking status and diabetes. A Cox-proportional hazard model was used to analyze whether immune responsiveness and circulating markers of inflammation are associated with mortality. The associations were adjusted for age, smoking status, diabetes and pravastatin allocation. In an additional analysis, we adjusted the association between markers of inflammation and mortality for the immune responsiveness composite score and we adjusted the association between immune responsiveness and mortality for the circulating markers of inflammation composite score. In order to account for a possible modulatory effect of pravastatin (Methe *et al.*, 2005; Niessner *et al.*, 2006; Shepherd *et al.*, 2002) the analyses were repeated stratified for subjects who received placebo (n = 205) and pravastatin (n = 198). Kaplan-Meier survival curves were used for visualization. For this visualisation we dichotomized the study population into a “High” and a “Low” immune responsiveness category based on the groups mean immune responsiveness composite score. The P-values for the Kaplan-Meier survival curves were calculated using log-rank tests. All analyses were performed using SPSS software version 20.0 (IBM, Armonk, New York, USA) and StataCorp Stata/SE version 12.0. P-values < 0.05 were regarded as statistically significant.

4.3 Results

Table 4.1. Baseline characteristics.

Characteristic	Study population n=403
Age, years	75.1 (3.3)
Sex, % men	53.6
BMI, kg/m²	26.9 (3.6)
Current smokers, %	23.8
Comorbidities	
Hypertension, %	65.5
Diabetes, %	19.6
History of myocardial infarction, %	13.2
History of stroke or TIA, %	15.1
History of vascular disease, %	44.9
Laboratory measurements	
Total cholesterol, mmol/L	5.8 (0.8)
HDL cholesterol, mmol/L	1.3 (0.3)
Circulating markers of chronic inflammation, median (IQR)	
Hs-CRP, mg/L,	2.9 (1.7-5.2)
Serum IL-6, pg/mL	2.7 (2.0-4.1)
Immune responsiveness[#], median (IQR)	
IL-6, pg/ml	71351 (54884-95568)
TNF-α, pg/ml	14334 (9199-22279)
IL-12, pg/ml	12601 (8102-19720)
IL-1β, pg/ml	8105 (5208-13208)
IL-10, pg/ml	1573 (1115-2238)
IL-1RA, pg/ml	35918 (27423-50975)

Data are presented as mean (SD) unless stated otherwise. BMI: body mass index; TIA: transient ischemic attack; HDL: high-density lipoprotein; Hs-CRP: high-sensitivity C-reactive protein; IQR: Interquartile range. [#] cytokine production response upon whole-blood stimulation with lipopolysaccharide.

Table 4.1 shows the baseline characteristics in the study population including 403 subjects. The mean age was 75.1 years (SD 3.3) and 53.6% of the population was men. The median follow-up time was 12.8 years (interquartile range: 12.8–12.9). In this period, 217 subjects (53.8%) died. Sixty-six subjects (16.3%) died due to a cardiovascular cause and 148 sub-

jects (36.7%) died due to a non-cardiovascular cause. For three subjects (1.4%) the cause of death was not recorded.

Table 4.2. Correlation between circulating markers of inflammation and immune responsiveness at baseline.

	Circulating markers of inflammation		
Immune responsiveness[#]	Hs-CRP *r*	Serum IL-6 *r*	Composite score *r*
IL-6	0.08	-0.01	0.04
TNF-α	-0.01	-0.04	-0.02
IL-12	-0.02	-0.09	-0.06
IL-1β	-0.02	-0.02	-0.02
IL-10	0.07	0.08	0.09
IL-1RA	0.24**	0.19**	0.25**
Composite score	0.08	0.01	0.05

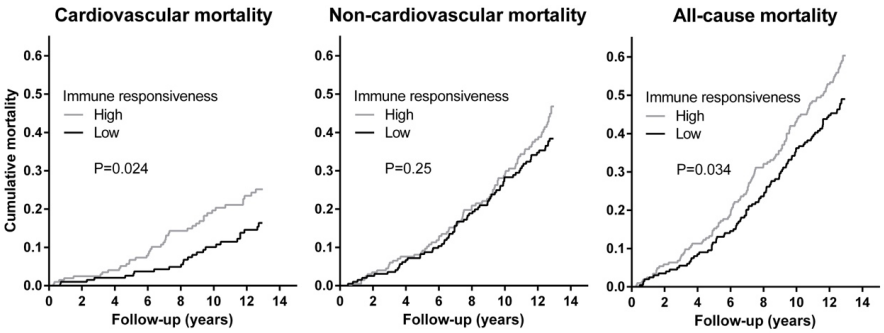
r: Correlation coefficient corrected for sex, age, smoking and diabetes; Immune responsiveness was determined by measuring cytokine production response upon whole-blood stimulation with lipopolysaccharide. Immune responsiveness composite score: the average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness; Circulating markers of inflammation composite score: the average sex-specific Z-score of Hs-CRP and serum IL-6; **P<0.001.

Table 4.2 shows the correlation between circulating markers of inflammation and IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness. None of the correlations were significant, except that IL-1RA production responsiveness was significantly positively correlated with Hs-CRP, serum IL-6 and the circulating markers of inflammation composite score.

Table 4.3. Mortality risks dependent on circulating markers of inflammation or immune responsiveness.

	Cardiovascular mortality		Non-cardiovascular mortality		All-cause mortality	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Circulating markers of inflammation						
Hs-CRP, per SD increment	1.21 (0.95-1.53)	0.13	1.11 (0.94-1.31)	0.22	1.13 (0.99-1.29)	0.067
Serum IL-6, per SD increment	1.18 (0.95-1.47)	0.14	**1.22 (1.05-1.42)**	**0.008**	**1.21 (1.07-1.37)**	**0.002**
Composite score, per SD increment	1.25 (0.97-1.62)	0.086	**1.21 (1.02-1.44)**	**0.030**	**1.22 (1.06-1.41)**	**0.006**
Immune responsiveness[#]						
IL-6, per SD increment	**1.50 (1.12-2.00)**	**0.003**	1.16 (0.97-1.40)	0.11	**1.26 (1.08-1.47)**	**0.003**
TNF-α, per SD increment	**1.32 (1.03-1.69)**	**0.031**	1.11 (0.94-1.32)	0.22	**1.16 (1.01-1.33)**	**0.036**
IL-12, per SD increment	1.25 (0.94-1.67)	0.12	1.06 (0.89-1.28)	0.51	1.11 (0.95-1.29)	0.19
IL-1β, per SD increment	**1.41 (1.07-1.87)**	**0.015**	1.14 (0.95-1.37)	0.17	**1.20 (1.03-1.40)**	**0.018**
IL-10, per SD increment	1.09 (0.78-1.54)	0.61	1.15 (0.92-1.44)	0.23	1.13 (0.94-1.36)	0.20
IL-1RA, per SD increment	**1.41 (1.09-1.83)**	**0.010**	1.08 (0.90-1.30)	0.38	**1.17 (1.01-1.36)**	**0.036**
Composite score, per SD increment	**1.89 (1.26-2.85)**	**0.002**	1.24 (0.95-1.63)	0.11	**1.39 (1.12-1.74)**	**0.003**

Values in bold are statistically significant (P<0.05). Results are presented as hazard rates (HR) with corresponding confidence intervals (95% CI) and were adjusted for age, diabetes, smoking and pravastatin allocation. Circulating markers of inflammation composite score: the average sex-specific Z-score of Hs-CRP and serum IL-6. [#] cytokine production response upon whole-blood stimulation with lipopolysaccharide. Immune responsiveness composite score: the average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness.



Immune responsiveness: The average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production response upon whole-blood stimulation with lipopolysaccharide. High: above the groups mean, Low: below the groups mean; P-values were calculated using log-rank tests.

Figure 4.1. Kaplan-Meier curves.

Table 4.3 shows the Cox-proportional hazard analyses of circulating markers of inflammation and immune responsiveness with mortality. Serum IL-6 and the circulating markers of inflammation composite score were significantly positively associated with non-cardiovascular and all-cause mortality, but not with cardiovascular mortality. IL-6, TNF-α, IL-1β, and IL-1RA production responsiveness and the immune responsiveness composite score were significantly positively associated with cardiovascular and all-cause mortality, but not with non-cardiovascular mortality. Figure 4.1 depicts Kaplan-Meier curves of subjects with a high or a low immune responsiveness composite score in relation to cardiovascular, non-cardiovascular and all-cause mortality.

Table 4.4 shows the reciprocal adjustment of circulating markers of inflammation and immune responsiveness on the relation with mortality. Results did not change significantly. Supplementary table 4.5 and 4.6 show that the relation between immune responsiveness and cardiovascular mortality was more pronounced in subjects with placebo allocation compared to subjects with pravastatin allocation. However this difference was not statistically significant (p for interaction = 0.22).

Table 4.4. Reciprocally adjusted mortality risks dependent on markers of inflammation and immune responsiveness.

	Cardiovascular mortality		Non-cardiovascular mortality		All-cause mortality	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Circulating markers of inflammation*						
Hs-CRP, per SD increment	1.18 (0.93-1.51)	0.18	1.10 (0.93-1.30)	0.25	1.12 (0.98-1.28)	0.097
Serum IL-6, per SD increment	1.20 (0.96-1.50)	0.10	**1.23 (1.06-1.42)**	**0.006**	**1.22 (1.08-1.39)**	**0.001**
Composite score, per SD increment	1.25 (0.97-1.62)	0.089	**1.21 (1.02-1.44)**	**0.031**	**1.22 (1.06-1.41)**	**0.007**
Immune responsiveness°#						
IL-6, per SD increment	**1.50 (1.13-2.00)**	**0.005**	1.17 (0.97-1.40)	0.099	**1.26 (1.08-1.47)**	**0.003**
TNF-α, per SD increment	**1.34 (1.05-1.72)**	**0.021**	1.13 (0.96-1.34)	0.15	**1.18 (1.03-1.36)**	**0.018**
IL-12, per SD increment	1.28 (0.97-1.70)	0.86	1.08 (0.90-1.30)	0.41	1.13 (0.97-1.31)	0.12
IL-1β, per SD increment	**1.41 (1.07-1.87)**	**0.014**	1.15 (0.96-1.38)	0.13	**1.21 (1.04-1.41)**	**0.012**
IL-10, per SD increment	1.08 (0.78-1.55)	0.60	1.17 (0.93-1.47)	0.19	1.15 (0.95-1.38)	0.16
IL-1RA, per SD increment	**1.37 (1.05-1.80)**	**0.022**	1.04 (0.86-1.25)	0.72	1.13 (0.96-1.31)	0.13
Composite score, per SD increment	**1.90 (1.26-2.86)**	**0.002**	1.26 (0.97-1.65)	0.085	**1.41 (1.13-1.76)**	**0.002**

Values in bold are statistically significant (P<0.05). Results are presented as hazard rates (HR) with corresponding 95% confidence intervals (CI) and were adjusted for age, diabetes, smoking and pravastatin allocation. * additionally adjusted for immune responsiveness composite score. ° additionally adjusted for circulating markers of inflammation composite score. # cytokine production response upon whole-blood stimulation with lipopolysaccharide. Circulating markers of inflammation composite score: the average sex-specific Z-score of Hs-CRP and serum IL-6. Immune responsiveness composite score: the average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness;

4.4 Discussion

Higher IL-6, TNF-α, and IL-1β production responsiveness and a higher immune responsiveness composite score were associated with a significantly higher risk of cardiovascular and all-cause mortality. Adjusting these relations for the circulating markers of inflammation did not change these results.

In the present study we measured immune responsiveness in whole blood samples using a highly standardized stimulation assay, known to be a well-suited low-cost proxy-measure of monocytic cytokine production responsiveness (Damsgaard *et al.*, 2009a; Van der Linden *et al.*, 1998). Therefore, our results suggest that monocytes are an important source of the cytokines that are involved in cardiovascular mortality. Interestingly, monocytes are known to be recruited into atherosclerotic plaques and to be involved in the remodelling of cardiac tissue after a myocardial infarction (Nahrendorf, Pittet & Swirski, 2010; Randolph, 2013). Immune responsiveness differs from circulating markers of inflammation, since these circulating markers have as their source a wide variety of cell types including lymphoid cells as well as non-lymphoid cells like endothelial cells, fibroblasts and adipocytes (Naka, Nishimoto & Kishimoto, 2002). Circulating markers of inflammation are therefore a reflection of the systemical inflammatory status. Immune responsiveness reflects the ability of blood cells to produce, when challenged by infectious agents, high amounts of cytokines within 24 hours. We used the stimulus LPS which is relevant in relation to the observation that there is a transient increase in risk for a vascular event after an infection (Smeeth *et al.*, 2004).

Table 4.5. Circulating markers of chronic inflammation and cytokine production response in relation to mortality in the placebo group.

	Cardiovascular mortality		Non-cardiovascular mortality		All-cause mortality	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Circulating markers of inflammation						
Hs-CRP, per SD increment	1.18 (0.86-1.63)	0.30	1.24 (0.98-1.55)	0.070	**1.22 (1.01-1.47)**	**0.037**
Serum IL-6, per SD increment	1.17 (0.89-1.55)	0.26	**1.22 (1.00-1.49)**	**0.049**	**1.21 (1.02-1.42)**	**0.024**
Composite score, per SD increment	1.24 (0.88-1.75)	0.21	**1.29 (1.02-1.65)**	**0.036**	**1.28 (1.05-1.55)**	**0.015**
Immune responsiveness[#]						
IL-6, per SD increment	**1.90 (1.25-2.89)**	**0.003**	1.08 (0.81-1.43)	0.60	**1.30 (1.03-1.64)**	**0.028**
TNF-α, per SD increment	**1.43 (1.02-2.00)**	**0.036**	1.03 (0.81-1.30)	0.83	1.15 (0.95-1.39)	0.15
IL-12, per SD increment	1.40 (0.95-2.06)	0.085	0.91 (0.71-1.18)	0.49	1.05 (0.85-1.29)	0.66
IL-1β, per SD increment	1.42 (0.98-2.04)	0.060	1.04 (0.82-1.34)	0.73	1.15 (0.94-1.41)	0.17
IL-10, per SD increment	1.39 (0.87-2.21)	0.16	1.12 (0.80-1.57)	0.52	1.21 (0.92-1.58)	0.18
IL-1RA, per SD increment	1.37 (0.96-1.97)	0.086	1.21 (0.93-1.57)	0.17	**1.26 (1.02-1.56)**	**0.033**
Composite score, per SD increment	**2.46 (1.36-4.44)**	**0.003**	1.12 (0.75-1.66)	0.58	**1.45 (1.04-2.00)**	**0.026**

Values in bold are statistically significant (P<0.05). Results are presented as hazard rates (HR) with corresponding 95% confidence intervals (CI) and were adjusted for age, diabetes and smoking. [#] cytokine production response upon whole-blood stimulation with lipopolysaccharide. Immune responsiveness composite score: the average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness. Circulating markers of inflammation composite score: the average sex-specific Z-score of Hs-CRP and serum IL-6.

Our data suggests that the transiently increased risk for vascular events may be related to immune responsiveness of blood cells, although this needs to be further investigated. The stimulus LPS is an agonist of toll-like receptor 4 (TLR-4), which is known to recognize also endogenous danger signals like high-mobility group box-1 (HMGB-1) (Bianchi & Manfredi, 2009). HMGB-1 is released by necrotic cells and acts as an early mediator of inflammation and organ damage in ischemia/reperfusion damage of the heart (Andrassy *et al.*, 2008). Therefore, the immune response upon stimulation with LPS could also be seen as a proxy for the immune response upon a myocardial infarction. This could be an explanation for the finding that immune responsiveness was associated with cardiovascular mortality and this association was independent of circulating markers of inflammation which are associated with cardiovascular mortality via different pathophysiolical mechanisms like the formation of atherosclerosis (Sprague & Khalil, 2009).

Our study was performed in a clinical trial population at risk for cardiovascular disease with a mean age of 75 years. The findings from this study are different from a population based study in elderly aged 85 years showing an higher risk for all-cause mortality in subjects with a low pro- and low anti-inflammatory immune responsiveness (Wijsman *et al.*, 2011). Probably, a low immune responsiveness is in this population of oldest old more a reflection of an age related impaired immune function and that this increased the risk for fatal infections (Bruunsgaard *et al.*, 1999; Van den Biggelaar *et al.*, 2004). In line with our findings, a higher TNF-α production responsiveness has in this population been related with a higher risk of cardiovascular

Table 4.6. Mortality risks dependent on circulating markers of inflammation and immune responsiveness in subjects with pravastatin allocation.

	Cardiovascular mortality		Non-cardiovascular mortality		All-cause mortality	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Circulating markers of inflammation						
Hs-CRP, per SD increment	1.23 (0.85-1.78)	0.27	1.35 (0.99-1.83)	0.056	1.06 (0.87-1.29)	0.54
Serum IL-6, per SD increment	1.19 (0.83-1.71)	0.34	1.00 (0.79-1.27)	0.99	**1.21 (1.01-1.46)**	**0.041**
Composite score, per SD increment	1.26 (0.85-1.86)	0.25	1.21 (0.97-1.50)	0.091	1.17 (0.95-1.44)	0.14
Immune responsiveness[#]						
IL-6, per SD increment	1.17 (0.78-1.77)	0.45	1.13 (0.88-1.46)	0.34	1.23 (1.00-1.51)	0.053
TNF-α, per SD increment	1.17 (0.79-1.75)	0.43	1.21 (0.95-1.58)	0.092	1.17 (0.95-1.43)	0.14
IL-12, per SD increment	1.08 (0.70-1.67)	0.72	1.26 (0.96-1.65)	0.094	1.18 (0.94-1.48)	0.15
IL-1β, per SD increment	1.40 (0.91-2.16)	0.13	1.28 (0.97-1.67)	0.075	**1.28 (1.02-1.61)**	**0.033**
IL-10, per SD increment	0.81 (0.48-1.35)	0.41	1.25 (0.91-1.73)	0.17	1.07 (0.82-1.38)	0.63
IL-1RA, per SD increment	1.43 (0.98-2.09)	0.067	0.78 (0.74-1.25)	0.78	1.10 (0.89-1.35)	0.39
Composite score, per SD increment	1.41 (0.77-2.56)	0.26	1.40 (0.97-2.01)	0.073	1.35 (0.99-1.83)	0.056

Values in bold are statistically significant (P<0.05). Results are presented as hazard rates (HR) with corresponding 95% confidence intervals (CI) and were adjusted for age, diabetes and smoking. Circulating markers of inflammation composite score: the average sex-specific Z-score of Hs-CRP and serum IL-6. [#]cytokine production response upon whole-blood stimulation with lipopolysaccharide. Immune responsiveness composite score: the average sex-specific Z-score of IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA production responsiveness.

mortality (Van den Biggelaar *et al.*, 2004). However, in the present study no significant relation between IL-10 production responsiveness and cardiovascular mortality was found, while in these elderly aged 85 years it has been shown that a higher IL-10 production responsiveness associates with a lower risk for fatal stroke and cardiovascular mortality (Van Exel *et al.*, 2002; Van den Biggelaar *et al.*, 2004). An explanation could be that in the latter population the heterogeneity among subjects was higher due to their high age and due to the absence of selection criteria for health characteristics in the study.

Although immune responsiveness, determined by measuring cytokine production response upon whole-blood stimulation with LPS, is over 50% genetically determined, recent studies showed that infections and vaccinations not only challenge cytokine production responsiveness acutely, but also impacts the epigenetic reprogramming of monocytes (Kleinnijenhuis *et al.*, 2012; Quintin *et al.*, 2012). Bacille Calmette-Guerin (BCG), the vaccine against tuberculosis, induces in men an increased immune response upon *ex-vivo* stimulation with unrelated microbial products for at least three months (Kleinnijenhuis *et al.*, 2012). A similar prolonged changed immune responsiveness was observed by *Candida albicans* infection in mice (Quintin *et al.*, 2012). These findings suggest that immune responsiveness can be a novel target for the prevention of cardiovascular diseases (Bekkering *et al.*, 2013).

In conclusion, within elderly at risk for cardiovascular disease, we showed that immune responsiveness is associated with cardiovascular mortality and all-cause mortality independent of circulating markers of inflammation. Immune responsiveness may be a target for future therapy and screening pa-

tients for immune responsiveness may contribute to a better cardiovascular mortality risk prediction.

Chapter 5

Men have higher whole blood cytokine production responses than women: a pooled-analysis including 15 study populations

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Abstract

Incidence and prognosis of infectious diseases, sepsis, and auto-immune diseases differ between men and women. These differences have been attributed to sex differences in immune responsiveness. However, results from studies investigating sex differences in whole blood cytokine production response upon *ex-vivo* stimulation with lipopolysaccharide (LPS), a widely used assay for immune responsiveness, are inconclusive. The aim of the present study is to investigate sex differences in whole blood cytokine production response using cytokine data from 4020 subjects originating from 15 study populations, either from the general population or from patient populations with specific diseases. Men compared to women had a higher cytokine production response for tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-12, IL-1β, IL-1RA, IL-10, but not for interferon-γ. Granulocyte macrophage colony-stimulating factor production response was lower in men compared to women. These sex differences were independent of chronological age. As men had higher monocyte concentrations, we normalized cytokine production responses for monocyte concentration. After normalization sex differences in cytokine production response disappeared, except for IL-10, of which the production response was lower in men compared to women. In conclusion, men have higher whole blood cytokine production responses than women, which are largely explained by higher monocyte concentrations. A sex-based approach of handling immune responsiveness is crucial.

5.1 Introduction

Although incidence of bacterial infections (Klein, 2012; Washburn, Medearis & Childs, 1965; Offner, Moore & Biffl, 1999) and sepsis (Dombrovskiy *et al.*, 2007) are higher in men than in women, men less often develop auto-immune diseases (Beeson, 1994; Whitacre, 2001; Jacobson *et al.*, 1997) and have lower mortality risks in sepsis (Pietropaoli *et al.*, 2010; Nachtigall *et al.*, 2011; McLauchlan *et al.*, 1995; Eachempati, Hydo & Barie, 1999; Wichmann *et al.*, 2000; Schroder *et al.*, 1998). These clinical findings have been attributed to sex differences in immune responsiveness, which is hypothesized to be the evolutionary consequence of sex differences in reproductive strategies (Fish, 2008; McKean & Nunney, 2005).

Immune responsiveness can be assessed by a whole blood stimulation assay which measures the amount of cytokines produced by immune cells

upon stimulation with lipopolysaccharide (LPS) (Desch *et al.*, 1989; De Groote *et al.*, 1992). This stimulation assay is well reproducible (Van der Linden *et al.*, 1998), assesses primarily subjects cytokine production by monocytes (Damsgaard *et al.*, 2009a), which is under tight genetic control (De Craen *et al.*, 2005). A higher pro-inflammatory cytokine and a lower anti-inflammatory production response have been associated with survival from meningococcal infection (Preuss *et al.*, 2003), lower incidence of systemic lupus erythematosus (Van der Linden *et al.*, 2000), and less severe progression of multiple sclerosis (De Jong *et al.*, 2000, 2002). Several studies have reported a higher cytokine production response in men compared to women (Aulock *et al.*, 2006; Bruunsgaard *et al.*, 1999; Lefevre *et al.*, 2012; Kim-Fine *et al.*, 2012; Heesen *et al.*, 2002), although others have found no sex differences (Lynch, Dinarello & Cannon, 1994; Moxley *et al.*, 2004). Furthermore, these sex differences were only found in young and healthy subjects (Aulock *et al.*, 2006; Bruunsgaard *et al.*, 1999; Lefevre *et al.*, 2012; Kim-Fine *et al.*, 2012; Heesen *et al.*, 2002) and not in elderly (Bruunsgaard *et al.*, 1999; Moxley *et al.*, 2004). Moreover, no sex differences were found in studies in patients with specific diseases, such as patients after elective coronary stent placement (Rittersma *et al.*, 2005), severe blunt trauma (Majetschak *et al.*, 2000), or before and after abdominal surgery (Scheingraber *et al.*, 2005). However, all studies were performed with a small number of subjects and investigated only a limited number of cytokines which hampers a conclusive and comprehensive interpretation of sex differences in whole blood cytokine production response.

In the last 18 years the assay to measure whole blood cytokine production response has been performed in the same laboratory on a standardized manner using the same techniques for 15 study populations. This allowed us to perform a meta-analysis on sex differences in whole blood cytokine production response in an unprecedented large number of subjects. The included subjects were young, middle aged or elderly from the general population or had specific diseases like osteoarthritis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, or cardiac diseases warranting cardiac surgery. We first investigated the cytokine production response for tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-12, IL-1β, IL-1RA, IL-10, interferon-γ (IFN-γ) and granulocyte macrophage colony-stimulating factor (GM-CSF) in men compared to women. Second, we investigated whether sex differences in cytokine production response were different in various age groups. Third, we explored whether observed differences in cytokine produc-

tion response between men and women were attributable to differences in monocyte concentrations.

5.2 Methods

5.2.1 Study design

We included 15 study populations for which data on cytokine production response were generated in our laboratory over the last 18 years. Subject characteristics and key references for the study populations are given in 5.1.

5.2.2 Cytokine production response

Cytokine production response was measured using whole blood samples upon stimulation with LPS as described elsewhere Desch *et al.* (1989); Van der Linden *et al.* (1998). In short, venous blood samples were drawn and collected in heparinized tubes. Samples were then diluted with RPMI-1640 and incubated after addition of *E.Coli*-derived LPS at 37˚C and 5% CO_2 for 24 hours. Table 5.2 shows the used LPS concentration and period of incubation for each study population. After centrifugation, the supernatants were stored at −80˚C until assayed for TNF-α, IL-6, IL-1β, IL-10, and IFN-γ using Pelikine compact ELISA kits (Sanquin, Amsterdam, The Netherlands). IL-12, IL-1RA and GM-CSF were assayed using ELISA kits of R&D systems (Minneapolis, MN, USA). Before 2010 for IL-12 and IL-1RA ELISA kits of BioSource (Louvain, Belgium) were used. Table 5.2 shows the availability of the production response of each cytokine and the key references for additional available information on the material and methods for each study population. Subjects with an unstimulated TNF-α concentration above 100 μg/mL were excluded from further analysis due to concerns of possible contamination or acute infection (n = 27).

5.2.3 Hematologic measurements

Leukocyte, neutrophil, lymphocyte, monocyte, eosinophil and basophil concentrations were measured in venous blood samples using a Sysmex XE-2100 Hematology Analyzer (Sysmex, Etten-Leur, the Netherlands). Table 5.2 shows for which study populations these hematologic measurements were available.

5.2.4 Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics version 20 and StataCorp Stata/SE version 12.0. For each subject cytokine production response values were natural log (ln) transformed due to skewness. For each study population the difference in cytokine production response between men and women was calculated using linear regression analyses with log-transformed cytokine production response as dependent variable and sex and age as independent variables. Some study populations included subjects from the same family (Table 5.1). Linear regression analyses of data from these study populations were adjusted for family relationships between subjects by use of a family clustering factor as a sandwich estimator.

Random effect meta-analyses were performed to estimate the overall log-transformed difference in cytokine production response between men and women. The overall ratio of men over women in cytokine production response and its 95% confidence interval were calculated using an anti-log transformation of the overall log-transformed difference in cytokine production response between men and women and its 95% confidence interval (Bland & Altman, 1996).

Random effect meta-regression analyses were performed to investigate the effect of chronological age on the ratio of men over women in cytokine production response. These analyses were performed using study populations from the general population only so that the possible sex-specific effects of specific diseases and their treatments on age-related changes in cytokine production response could not affect our results. For these analyses each study population was stratified in age decades. The mean ratio of men over women in cytokine production response of pro-inflammatory cytokines was obtained by averaging the ratio of men over women in cytokine production response of TNF-α, IL-6, IL-12, and IL-1β. The mean ratio of men over women in cytokine production response of anti-inflammatory cytokines was obtained by averaging the ratio of men over women in cytokine production response of IL-1RA and IL-10. If data for a cytokine was missing, data on the available cytokines were used to calculate the mean ratio of men over women in cytokine production response.

To investigate the effect of normalization for monocyte concentration on sex differences in cytokine production response we normalized the levels of produced cytokines for the monocyte concentration. Normalization was performed by dividing the cytokine level in pg/L by the monocyte concentration in count $\times 10^9$/L.

5.3 Results

Table 5.1 shows the characteristics of the included study populations. Ten study populations included subjects from the general population (n = 3114) and five study populations included subjects with specific diseases (n = 906). The mean age of the subjects of the various study populations ranged from 21 to 85 years. The age of the individual subjects ranged from 3 to 95 years. The mean percentage of men of the study populations ranged from 18.6% to 74.2%.

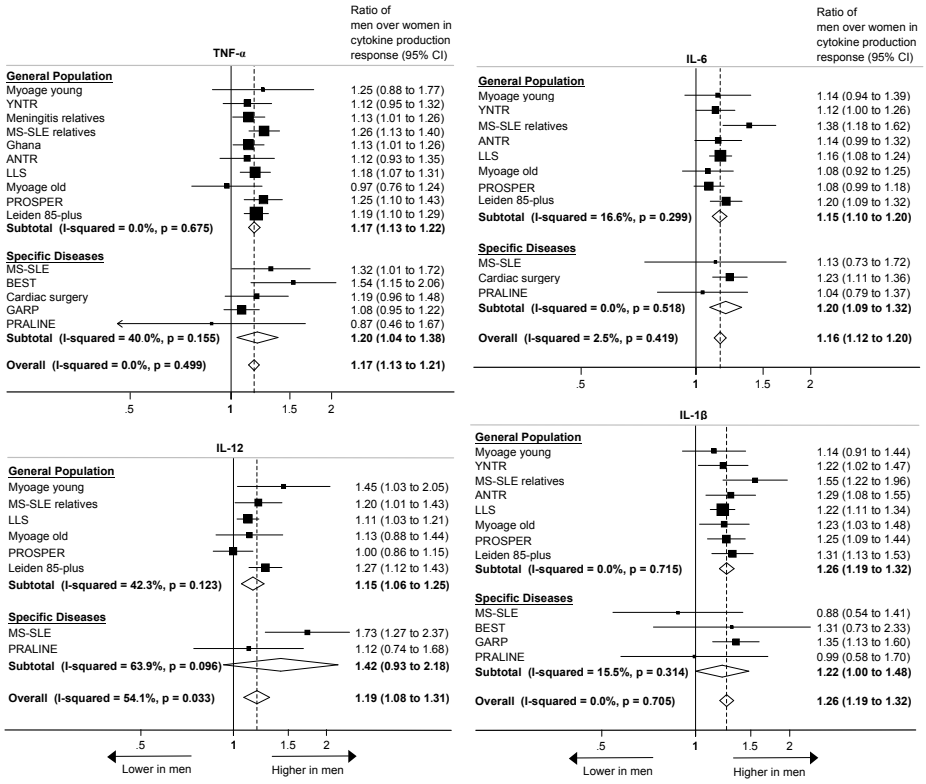

Figure 5.1. Forest plots of ratios of men over women in cytokine production response of pro-inflammatory cytokines.

Figure 5.1 shows forest plots of the ratios of men over women in cytokine production response for the pro-inflammatory cytokines TNF-α, IL-6, IL-12, and IL-1β. The cytokine production response of all cytokines was higher in men compared to women (all p-values < 0.001). Figure 5.2 shows the forest

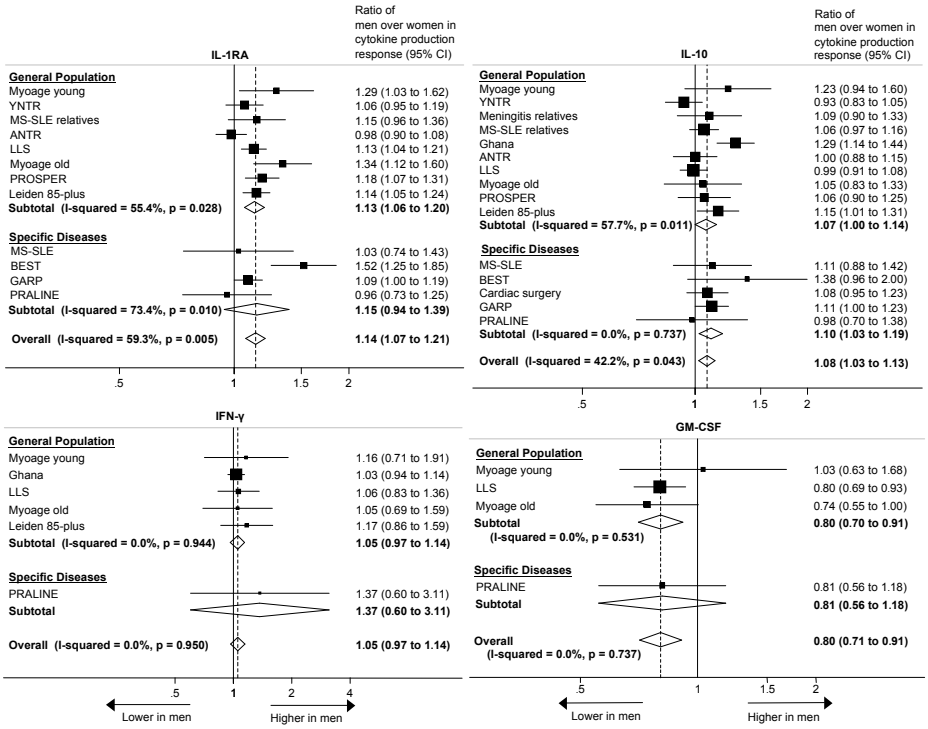

Figure 5.2. Forest plots of ratios of men over women in cytokine production response of anti-inflammatory and cytokines that stimulate pro-inflammatory cytokine production.

plots of the ratios of men over women in cytokine production response for the anti-inflammatory cytokines IL-1RA and IL-10 and the cytokines that stimulate pro-inflammatory cytokine production, IFN-γ and GM-CSF. The cytokine production response of IL-1RA and IL-10 was higher in men compared to women (p-values < 0.001 and < 0.05 respectively). No significant sex differences in cytokine production response of IFN-γ were found (p-value $= 0.23$). The cytokine production response of GM-CSF was lower in men compared to women (p-values < 0.001). Results from study populations including subjects from the general population were not significantly different from results from that including subjects with specific diseases. Noteworthy, an identical pattern was observed in the Ghana study including subjects from African decent living under adverse environmental conditions in Ghana and in studies including subjects from Western populations.

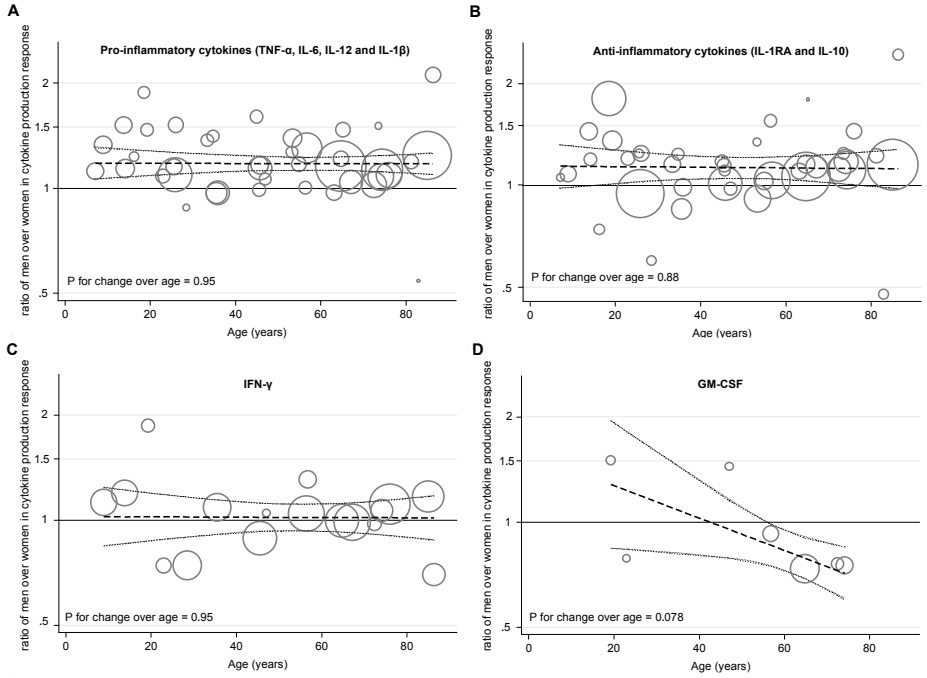

Figure 5.3. Ratios of men over women in cytokine production response dependent on chronological age.
Scatter plot of study populations from the general population stratified for age decades with meta-regression lines with 95% confidence interval. A larger circle size indicates a higher precision of the estimate (circle size = $1/SE^2$). An estimate represents the mean of the ratio of men over women in cytokine production response for (**A**) TNF-α, IL-6, IL-12 and IL-1β (n = 40), (**B**) IL-1RA and IL-10 (n = 40), (**C**) IFN-γ (n = 17), and (**D**) GM-CSF (n = 7).

Figure 5.3 shows the mean ratios of men over women in cytokine production response over chronological age for the study populations including subjects from the general population. The mean ratios of men over women in cytokine production response for the pro-inflammatory (IL-6, TNF-α, IL-1β, and IL-12) and anti-inflammatory cytokines (IL-10 and IL-1RA) as well as for IFN-γ did not change over age (p-values for change over age all > 0.87). The mean ratio of men over women in cytokine production response for GM-CSF was lower at higher ages, but not statistically significant (p value for change over age = 0.078).

In three of included studies, Myoage young, Myoage old and LLS, it was

possible to explore whether sex differences in cytokine production response were attributable to sex differences in monocyte concentrations due to the availability of data on leukocyte differential concentrations. Table 5.3 shows the pooled leukocyte differential concentrations in men and women. Leukocyte concentrations did not differ between men and women. Neutrophil and monocyte concentrations were higher in men compared to women ($p = 0.008$ and < 0.001 respectively), while lymphocyte concentration was lower in men compared to women ($p < 0.001$). Therefore, cytokine production response of the cytokines that are mainly produced by monocytes (IL-6, TNF-α, IL-1β, IL-12, IL-10, and IL-1RA) were normalized for the monocyte concentration for each subject. Table 5.4 shows that the pooled ratios of men over women in cytokine production response normalized for monocyte concentration for IL-6, TNF-α, IL-12, IL-1β, and IL-1RA were not significantly different from unity, indicating the absence of sex differences. The pooled ratio of men over women in cytokine production response normalized for monocyte concentration for IL-10 was 0.89 (95% CI 0.83 to 0.96).

5.4 Discussion

In the present study we showed that men compared to women had a higher cytokine production response upon stimulation with LPS for IL-6, TNF-α, IL-12, IL-1β, IL-10, and IL-1RA, but not IFN-γ. GM-CSF production response was lower in men. These sex differences did not differ over age. As men had higher monocyte concentrations, we normalized cytokine production responses for monocyte concentration. No sex differences in cytokine production response per monocyte were found, except of IL-10 which was significantly lower in men.

Our findings are in agreement with other studies reporting a higher cytokine production response in men compared to women (Aulock *et al.*, 2006; Bruunsgaard *et al.*, 1999; Lefevre *et al.*, 2012; Kim-Fine *et al.*, 2012; Heesen *et al.*, 2002; Auger *et al.*, 2011; Balteskard, Brox & Osterud, 1993; Beenakker *et al.*, 2013; Kimura *et al.*, 2008; Trilok-Kumar *et al.*, 2012). However, these studies were small and found differences only in relatively young and healthy subjects. Moreover, others did not find sex differences (Bruunsgaard *et al.*, 1999; Lefevre *et al.*, 2012; Moxley *et al.*, 2004; Rittersma *et al.*, 2005; Majetschak *et al.*, 2000; Scheingraber *et al.*, 2005; Azevedo *et al.*, 2005). We showed that sex differences in cytokine production response are present in healthy populations of all ages and are independent of specific diseases

like osteoarthritis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, or cardiac diseases warranting cardiac surgery. Moreover, we found the same pattern of sex differences in Ghanaians from African decent living under adverse environmental conditions in Ghana. These findings confirm sex differences in cytokine production response as a true biological phenomenon.

The measurement of whole blood cytokine production response is a model primarily designed to investigate immune responsiveness in sepsis. In patients with multiple injuries, a higher TNF-? and IL-6 production response on the first day of admission has been associated with a higher risk of developing severe sepsis (Majetschak *et al.*, 2000). We showed that TNF-α and IL-6 production response are higher in men compared to women, which could be one of the factors causing the higher incidence of sepsis in men compared to women (Dombrovskiy *et al.*, 2007), next to other factors like sex differences in tissue derived cytokine production and antigen exposure (Klein, 2000; Van Eijk *et al.*, 2007). In contrast, mortality risks in sepsis patients have shown to be lower in men compared to women (Pietropaoli *et al.*, 2010; Nachtigall *et al.*, 2011; McLauchlan *et al.*, 1995; Eachempati, Hydo & Barie, 1999; Wichmann *et al.*, 2000; Schroder *et al.*, 1998). This could be explained by the observation that the majority of the deaths in sepsis do not occur during the initial hyperinflammatory phase, but during the later immunosuppressive phase characterized by a reduced pro-inflammatory cytokine production response (Hotchkiss *et al.*, 2013). IL-1β production response during the late phase of sepsis has been shown to be higher in sepsis survivors compered to non-survivors (Weighardt *et al.*, 2000). We found a higher IL-1β production response in men compared women. However, we did not investigate whether this sex difference is still present during the late immunosuppressive phase in sepsis patients. If so, then this could be one of the factors causing the lower sepsis mortality risk in men compared to women.

The measurement of cytokine production response upon stimulation with LPS has also been used as model of immune responsiveness in non-infectious disease. A low cytokine production response has, on the one hand, been associated with consequences of the aging process like mortality (Wijsman *et al.*, 2011), metabolic syndrome, type 2 diabetes (Van Exel *et al.*, 2002), lower serum triiodothyronine levels (Rozing *et al.*, 2011), and low muscle mass and strength (Beenakker *et al.*, 2013). On the other hand, a low cytokine production response has been associated with familial traits like a negative family history of Alzheimer's disease (Van Exel *et al.*, 2009), a less severe

progression of osteoarthritis within sibling pairs with osteoarthritis at multiple sites (Botha-Scheepers *et al.*, 2008; Bijsterbosch *et al.*, 2014), and a negative family history of systemic lupus erythematosus (Van der Linden *et al.*, 2000). It is tempting to speculate about the contribution of sex differences in immune responsiveness in the sex differences in incidences and prognoses of these diseases. However, it still needs to be elucidated whether cytokine production response plays a causal role in the development or progression of these diseases.

This study is the first to describe the relation between chronological age and sex differences in cytokine production response. Sex differences are commonly attributed to the sex hormones and are expected to be reduced after menopause. However, we found that sex differences in cytokine production response are independent of chronological age and remained after menopause. These results are in contrast with one relatively small study showing that sex differences in TNF-α cytokine production response are present in young subjects and not in elderly (Bruunsgaard *et al.*, 1999). Moreover, experimental studies on the effects of oestrogen, progesterone, and testosterone on cytokine production response report conflicting results (Bouman, Heineman & Faas, 2005). Likely, sex differences are not caused by the activational (reversible) effects of sex hormones on monocytes, but by the organizational (permanent) effects of sex hormones on monocyte concentrations in the blood (Arnold, 2009). Alternatively, sex differences could also be independent of sex hormones and be caused by the effect of a sex-biased expression of X and Y genes in bone marrow cells (Arnold, 2009).

We found that the observed sex differences in cytokine production response are largely dependent on sex differences in monocyte concentration. In agreement with our findings, some have reported higher monocyte concentrations in men compared to women (Aulock *et al.*, 2006; Starr & Deary, 2011; McIlhagger *et al.*, 2010; Bouman *et al.*, 2004), although others did not found sex differences (Bain & England, 1975; Bain, 1996). Like Aulock *et al.* (2006), normalization of cytokine production responses for individual monocyte concentration largely cancelled sex differences for the majority of the cytokines in our study. It is currently unknown whether normalization of cytokine production response for individual monocyte concentration increases the clinical relevance of the measurement. One could argue that it is not relevant whether the cytokines are produced by a larger number of monocytes or by a larger production of cytokines per monocyte (Van der Linden *et al.*, 1998). This argument is especially applicable for studies investigating

the effect of cytokine production response on other circulating immune cells like lymphocytes, since produced cytokines influence these cells directly via their cytokine receptors. The same is true for the effect of cytokine production response on vascular endothelial cells, which are also known to have cytokine receptors that are able to induce vascular dysfunction (Sprague & Khalil, 2009). However, the argument might be less applicable for studies investigating diseases like cancer where the monocytes infiltrate the tissue. In these cases, the cytokine production response is dependent on the number of monocytes available to infiltrate, and therefore normalising the cytokine production response for individual monocyte concentration would be preferred.

Interestingly, we found that the IL-10 production response per monocyte is higher in women compared to men. In women, reproductive success has been described to be dependent on a high IL-10 production response at the foetal-maternal interface (Piccinni *et al.*, 1998; Westendorp *et al.*, 2001). Monocytes infiltrate into the uterine tissues during early gestation and significantly increase in number with the onset of labor (Gomez-Lopez, Guilbert & Olson, 2010). However, it is not known whether reproductive success associates with the number of infiltrating monocytes independent of their IL-10 production response.

The present study has several strengths. First, data from all individual subjects were available. Second, a large variety of cytokines were measured in a large variety of study populations, including subjects within a broad age range and with different health conditions. Third, all cytokines were measured in our laboratory or in cooperation with our laboratory in a standardized manner. A limitation is that cytokine production response was measured using a specific ligand secreted by bacteria. However, also other pathogens as well as trauma elicit the same inflammatory response and TLR-4, the pathogen recognition receptors (PRR) for LPS, is also known to recognize endogenous danger signals (Bianchi & Manfredi, 2009).

In conclusion, we showed that sex differences in immune responsiveness and leukocyte differential concentrations are profound and consistent. This underscores the notion that the immune system of men and women are distinctly different and underscores the importance of taking sex into account when studying immunologic processes. We postulate that a sex-based approach intervening the immune system is crucial for immune modulatory therapies.

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Table 5.1. Characteristics of the included study populations.

Study name	Key ref. for study design	Subjects (n)	Family clusters (n)	Characteristics subjects	Age mean (SD)	Men (%)
General population						
Myoage young	McP13	35	n/a	Young subjects	21·4 (2·7)	42·9
YNTR	Boo06	161	74	Young twins and their first degree relatives	27·0 (4·1)	47·2
Meningitis relatives	Wes97	180	60	First degree relatives of meningitis patients	30·9 (16·0)	48·3
MS-SLE relatives	Lin00	550	177	First degree relatives of MS and SLE patients	44·8 (16·9)	44·5
Ghana	May09	627	n/a	Population living under adverse environmental conditions in Ghana	47·2 (20·6)	26·5
ANRT	Boo06	140	64	Adult twins and first degree relatives	49·6 (6·9)	45·0
LLS	Bee13	392	322	Offspring of long-lived sibling and the spouses of the offspring	63·0 (6·5)	49·7
Myoage old	McP13	74	n/a	Relatively healthy and cognitively active elderly	74·2 (3·1)	51·4
PROSPER	She99	403	n/a	Elderly at risk for cardiovascular diseases	75·1 (3·3)	53·6
Leiden 85-plus	Wie02	552	n/a	Inhabitants of Leiden aged 85 years	85 (-)	33·2
Specific diseases						
MS-SLE	Lin00	152	n/a	Patients with MS or SLE	46·2 (12·8)	28·3
BEST	Goe05	73	n/a	RA patients before start with DMARDs	55·1 (15·1)	32·9
Cardiac surgery	Lat09	199	n/a	Patients before cardiac surgery	64·3 (11·6)	72·4
GARP	Riy05	370	188	OA patients and OA-relatives with joint complains	70·7 (7·6)	18·6
PRALINE	Sch16	112	n/a	Patients before knee replacement surgery	73·1 (9·9)	18·8

MS: multiple sclerosis, SLE: systemic lupus erythematosus, OA: osteoarthritis, RA: rheumatoid arthritis, Number of family structures: number of independent observations corrected for family relationships between subjects, n/a: not available. **See section 5.5 for the key references.**

Table 5.2. Method of whole blood stimulation and availability of cytokine production capacity per study population.

Name study population	Key ref. for methods	LPS conc.	Monocyte conc. available	Cytokine production response							
				TNF-α	IL-6	IL-12	IL-1β	IL-1RA	IL-10	IFN-γ	GM-CSF
General population											
Myoage young	n/a	10 µg/ml	v	v	v	v	v	v	v	v	v
YNTR	Cra05	10 ng/ml		v	v		v	v	v		
Meningitis relatives	Wes97	1 µg/ml		v[†]					v		
MS-SLE relatives	Lin00	10 ng/ml		v	v	v	v	v	v		
Ghana	May09	10 µg/ml		v					v	v	
ANTR	Cra05	10 ng/ml		v	v		v	v	v		
LLS	Bee13	10 ng/ml	v	v	v	v[§]	v	v	v	v	v[§]
Myoage old	n/a	10 µg/ml	v	v	v	v	v	v	v	v	v
PROSPER	Tro09	10 ng/ml		v	v	v	v	v	v		
Leiden 85-plus	Big04	10 ng/ml		v*	v	v	v	v	v	v	
Specific diseases											
MS-SLE	Lin00	10 ng/ml		v*	v	v[‡]	v	v	v		
BEST	Vri07	10 ng/ml		v			v	v	v		
Cardiac surgery	n/a	10 ng/ml		v	v				v		
GARP	Bot08	10 ng/ml		v*			v	v	v		
PRALINE	n/a	10 µg/ml		v	v	v	v	v	v	v	v

Ref.: Reference, Conc.: concentration, LPS concentration: whole blood samples were incubated for with lipopolysaccharide for 24 hours at 37°C. n/a: not available. * LPS was incubated for 4 hours, [†] LPS was incubated for 6 hours, [‡] 1µg/ml LPS was used, [§] 50 µg/ml LPS was used. **See section 5.5 for the key references.**

Table 5.3. Pooled leukocyte concentration and differentiation in men and women of three independent study populations (Myoage young, Myoage old and LLS).

	Men	Women	
	mean (95% CI)	mean (95% CI)	p-value
Leukocytes $*10^9$/L	6.08 (5.34 to 6.82)	5.87 (4.89 to 6.85)	0.29
Neutrophils $*10^9$/L	3.58 (3.08 to 4.08)	3.25 (2.56 to 3.94)	0.008
Lymphocytes $*10^9$/L	1.79 (1.58 to 2.00)	1.99 (1.73 to 2.25)	<0.001
Monocytes $*10^9$/L	0.53 (0.49 to 0.57)	0.46 (0.41 to 0.50)	<0.001
Eosinophils $*10^9$/L	0.15 (0.15 to 0.17)	0.15 (0.13 to 0.17)	0.12
Basophils $*10^9$/L	0.03 (0.02 to 0.03)	0.03 (0.02 to 0.04)	0.25

Estimates were calculated using a meta-analysis model with random effects and corrected for age and family relationships between subjects, CI: confidence interval.

Table 5.4. Pooled effect of monocyte concentration normalization on ratios of men over women in cytokine production response in three independent study populations (Myoage young, Myoage old and LLS).

	Not normalized for monocyte concentration		Normalized for monocyte concentration	
	Ratio of men over women in cytokine production response (95% CI)	p-value	Ratio of men over women in cytokine production response (95% CI)	p-value
TNF-α	1.14 (1.02 to 1.28)	0.021	0.98 (0.86 to 1.13)	0.79
IL-6	1.15 (1.08 to 1.22)	<0.001	0.98 (0.90 to 1.06)	0.61
IL-12	1.14 (1.04 to 1.24)	0.007	1.09 (1.00 to 1.20)	0.053
IL-1β	1.21 (1.12 to 1.31)	<0.001	1.06 (0.98 to 1.14)	0.17
IL-1RA	1.22 (1.08 to 1.38)	0.002	1.05 (0.92 to 1.19)	0.47
IL-10	1.03 (0.92 to 1.13)	0.59	0.89 (0.83 to 0.96)	0.001

Estimates were calculated using a meta-analysis model with random effects and corrected for age and family relationships between subjects, CI: confidence interval.

Chapter 6

Pro-inflammatory capacity of classically activated monocytes relates positively to muscle mass and strength

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Abstract

Background: In mice, monocytes that exhibit a pro-inflammatory profile enter muscle tissue after muscle injury and are crucial for clearance of necrotic tissue and stimulation of muscle progenitor cell proliferation and differentiation. The aim of this study was to test if pro-inflammatory capacity of classically activated (M1) monocytes relates to muscle mass and strength in humans.
Methods: This study included 191 male and 195 female subjects (mean age 64.2 years and 61.9 years, respectively, standard deviation 6.4) of the Leiden Longevity Study. Pro-inflammatory capacity of M1 monocytes was assessed by ex vivo stimulation of whole blood with Toll-like receptor (TLR) 4 agonist lipopolysaccharide (LPS) and TLR-2/1 agonist tripalmitoyl-S-glycerylcysteine (Pam3Cys-SK4), both M1 phenotype activators. Cytokines that stimulate M1 monocyte response (IFN-γ and GM-CSF) as well as cytokines that are secreted by M1 monocytes (IL-6, TNF-α, IL-12 and IL-1β) were measured. Analyses were adjusted for age, height and body fat mass.
Results: Upon stimulation with LPS, the cytokine production capacity of IFN-γ, GM-CSF, and TNF-α was significantly positively associated with lean body mass, appendicular lean mass and handgrip strength in men, but not in women. Upon stimulation with Pam3Cys-SK4, IL-6, TNF-α and Il-1β was significantly positively associated with lean body mass and appendicular lean in women, but not in men.
Conclusions: Taken together, this study shows that higher pro-inflammatory capacity of M1 monocytes upon stimulation is associated with muscle characteristics and sex dependent.

6.1 Introduction

In mice, monocytes and macrophages are crucial for muscle regeneration. After skeletal muscle injury, monocytes with a pro-inflammatory profile migrate from the blood into the muscle tissue and differentiate into pro-inflammatory cytokine producing macrophages, which clear necrotic tissue and stimulate muscle progenitor proliferation (Arnold *et al.*, 2007; Tidball & Wehling-Henricks, 2007). Two days after injury, pro-inflammatory macrophages in mice change into macrophages with an anti-inflammatory phenotype to stimulate further differentiation of muscle progenitor cells and fusion of myotubes (Arnold *et al.*, 2007; Lu *et al.*, 2011a). Injection of ex vivo activated human

macrophages in a rat model for myocardial infarction has been demonstrated to accelerate vascularisation and tissue repair and improve cardiac remodelling and function (Leor *et al.*, 2006). Others showed in mice that loss of signal transducer and activator of transcription 1 (STAT-1) in bone marrow-derived cells increases the levels of pro-inflammatory cytokines in the skeletal muscle after injury and also accelerates muscle regeneration (Gao *et al.*, 2012). The pro-inflammatory role of monocytes and macrophages in human skeletal muscle has not been investigated yet.

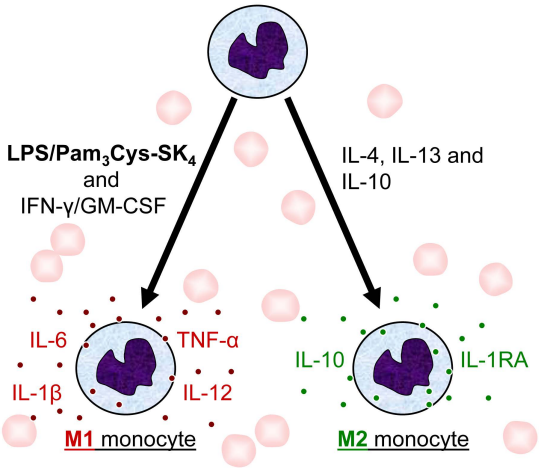

Figure 6.1. Cytokines that stimulate M1 and M2 monocyte response and cytokines, which are secreted by M1 and M2 monocytes. Monocytes stimulated ex-vivo with lipopolysaccharide (LPS) or tripalmitoyl-S-glycerylcysteine (Pam3Cys-SK4) differentiate into classically activated (M1) monocytes with a pro-inflammatory phenotype (Mills *et al.*, 2000; Wang *et al.*, 2007; Navarro-Xavier *et al.*, 2010). Exposure to interferon gamma (IFN-γ) or granulocyte/macrophage colony-stimulating factor (GM-CSF) has a priming and stimulatory effect on M1 monocytes (Kamijo *et al.*, 1993; Bundschuh *et al.*, 1997). Characteristic for M1 monocytes is the production of the pro-inflammatory cytokines interleukin (IL)-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-12, and IL-1β (Mantovani *et al.*, 2002). Monocytes differentiate into alternative activated monocytes (M2) with an anti-inflammatory phenotype upon stimulation with IL-13 and IL-4 alone, or together with IL-10 (Mantovani *et al.*, 2002). Characteristic for M2 monocytes is the production of the anti-inflammatory cytokines IL-1 receptor antagonist (IL-1RA) and IL-10 (Mantovani *et al.*, 2002).

In humans, a whole blood stimulation assay has been developed for the assessment of the capacity of a subject's monocytes to produce inflamma-

tory cytokines (Van Furth *et al.*, 1994; Damsgaard *et al.*, 2009a). Earlier studies have shown that the capacity to produce cytokines measured by this assay is highly reproducible (Van der Linden *et al.*, 1998) and under genetic control (De Craen *et al.*, 2005). Depending on the stimulant, monocytes and macrophages express a classically activated (M1) pro-inflammatory phenotype or an alternatively activated (M2) anti-inflammatory phenotype (as schematically depicted in Figure 6.1). In elderly humans, the capacity of monocytes to respond to M1 phenotype activators like toll-like receptor 4 (TLR-4) agonist lipopolysaccharide (LPS) and TLR-2/1 agonist tripalmitoyl-S-glycerylcysteine (Pam3Cys-SK4) (Mills *et al.*, 2000; Wang *et al.*, 2007; Navarro-Xavier *et al.*, 2010), has been reported to be lower compared to younger subjects (Ouyang *et al.*, 2000; Van den Biggelaar *et al.*, 2004; Nyugen *et al.*, 2010), although some found other results (Gabriel, Cakman & Rink, 2002). Whether cytokine production capacity of monocytes plays a role in age related loss of muscle mass and strength is unknown. Earlier we have shown that a pro-inflammatory cytokine production capacity is beneficial for survival in oldest old subjects (Wijsman *et al.*, 2011). We also found that tumor necrosis factor-α (TNF-α) production capacity relates to decline in handgrip strength in a selected group of subjects aged 85 years (Taekema *et al.*, 2007).

In the present study we further explored the relation between pro-inflammatory cytokine production capacity and skeletal muscle in a cohort of middle aged subjects. We stimulated whole blood ex-vivo with LPS or Pam3Cys-SK4 and assumed that the capacity to respond to LPS or Pam3Cys-SK4 is a proxy for the capacity to respond to stimuli from the skeletal muscle like muscle injury. The production capacity of two types of cytokines were assessed, namely those that are known to stimulate M1 monocyte response including interferon-γ (IFN-γ) and granulocyte macrophage colony-stimulating factor (GM-CSF) and those that are known to be secreted by M1 monocytes including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-12p40 (IL-12) and interleukin 1-β (IL-1β) (Mantovani *et al.*, 2002; Solinas *et al.*, 2009). We investigated whether the capacity to stimulate M1 monocyte response and the secretory capacity of M1 monocytes upon stimulation with LPS or Pam3Cys-SK4 are related to muscle mass and strength as an indicator for repair and maintenance of muscle during lifetime history in middle aged subjects.

6.2 Experimental Procedures

6.2.1 Study characteristics

The Leiden Longevity Study consists of offspring from long-lived Caucasian siblings and the partners thereof (Schoenmaker *et al.*, 2006). There were no selection criteria for health or demographic characteristics. Between November 2006 and May 2008, 392 subjects visited the study centre where measurements of innate immune capacity and muscle characteristics were performed. All measurements were carried out by trained research nurses. Information on medical history was obtained from the subjects' general practitioner (response 88.3%). Seven chronic diseases were documented including myocardial infarct, stroke, hypertension, diabetes mellitus, neoplasm, chronic obstructive disease and rheumatoid arthritis. Subjects diagnosed with leukaemia (n = 2) or use of oral corticosteroids (n = 4) were excluded from the analysis. The Medical Ethical Committee of the Leiden University Medical Centre approved the study and informed consent was obtained from all subjects.

6.2.2 Pro-inflammatory capacity of M1 monocytes

The pro-inflammatory capacity of M1 monocytes was assessed by measuring the cytokine production capacity of whole blood samples upon ex vivo stimulation with LPS or Pam3Cys-SK4 as describe elsewhere (Van Furth *et al.*, 1994; Van der Linden *et al.*, 1998). In short, cytokine production capacity was assessed by stimulating ex vivo using 2 ml of whole-blood. All venous blood samples were drawn in the morning before 11:00 am to exclude circadian variation. The blood was collected in heparinized tubes and samples were diluted two-fold with RPMI-1640 (Sigma, St. Louis, MO) and incubated after addition of 10 μg mL-1 or 50 μg mL-1 E. coli-derived LPS (Difco Laboratories, Detroit, MI) or 10 μg mL-1N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteinyl-(S)-seryl-(S)-lysyl-(S)-lysyl-(S)-lysyl-(S)-lysine, Pam3Cys-SK4, (EMC Microcollections, Tübingen, Germany) at 37˚C and 5% CO_2 for 24 hours. After centrifugation, the supernatants were stored at −80˚C until assayed for IFN-γ, GM-CSF, IL-6, TNF-α, IL-12 and IL-1β using standard ELISA techniques (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). IFN-γ, IL-6, TNF-α and IL-1β were measured in whole blood samples stimulated with 10 μg mL-1 LPS. GM-CSF and IL-12 were measured in whole blood samples stimulated with 50 μg mL-1 LPS. The

inter-assay cubic variance (CV) for the LPS stimulated cytokine assays, influenced also by dilutions for high cytokine values, were below 14% for IFN-γ; below 10% for GM-CSF and IL-6; below 18% for TNF-α and IL-1β and below 21% for IL-12. The inter-assay CV for Pam3Cys-SK4 stimulated cytokine assays were below 5% for IL-6, below 15% for TNF-α; below 10% for IL-12 and below 19% for IL-1β. The intra-assay CV ranged up to 15%. Cytokine levels outside the range of three standard deviations were regarded as outliers and exclude from the analyses (0 to 4 values per cytokine).

6.2.3 Hematologic measurements

Leukocyte and monocyte concentration was measured in venous blood samples using a Sysmex XE-2100 Hematology Analyzer (Sysmex, Etten-Leur, The Netherlands).

6.2.4 Body composition

Next to measurements of body weight and height, a Direct Segmental Multifrequency Bioelectrical Impedance Analysis (DSM-BIA) was performed using the In-Body (720) body composition analyser (GE healthcare, Madison, WI). Previously we have shown this technique to be a valid tool for the assessment of whole body composition and segmental lean mass measurements in our study population (Ling *et al.*, 2011). A tetrapolar eight-point tactile electrode system was used, which separately measures impedance of the subject's trunk, arms and legs at six different frequencies (1, 5, 50, 250, 500, 1000 kHz) for each of the body segment. Total body water (TBW) was estimate from area, volume, length, impedance and a constant proportion (specific resistivity). Lean body mass was estimated as TBW/0.73. Body fat mass was calculated as the difference between TBW and lean body mass. Appendicular lean mass calculation was based on lean mass of both the right and left arms and legs. Subjects wore normal indoor clothing and were asked to stand barefoot on the machine platform with their arms abducted and hands gripping on to the handles. Data on body composition measurement were unavailable in two subjects.

6.2.5 Handgrip strength

Handgrip strength of the dominant side was measured (to the nearest kilogram) using a JAMAR hand dynamometer (Sammons Preston Inc., Boling-

brook, IL) with the subject in an upright position. Subjects were advised to exert maximal force and one test trial was allowed, followed by three test measurements. The best measure recorded was taken for the final analysis.

6.2.6 Physical activity

Physical activity was self-reported using the Dutch version of the International Physical Activity Questionnaire (IPAQ) Short Version. IPAQ includes questions about hours per week spent in vigorous, moderate, slow activity, walking and sitting during the last seven days. Only data on hours of vigorous activity per week were used because validation studies have shown that these data best correlates with objectively measured physical activity (Lee *et al.*, 2011).

6.2.7 Statistical analyses

The Statistical Package for the Social Sciences (SPSS) program version 20.0 (IBM, Armonk, New York) was used for data analysis. Cytokine production capacity values were natural log transformed due to skewness. The production capacity of cytokines that stimulate M1 monocyte response and that are secreted by M1 monocytes were calculated based on sex specific Z-scores of each cytokine. This was performed to be able to compare effect-sizes of the different cytokine production capacities and to combine them in composite scores. A composite score was defined as the mean of the Z-scores of production capacity of cytokines that stimulate M1 monocytes response (IFN-γ and GM-CSF) or that are secreted by M1 monocytes (IL-6, TNF-α, IL-12, IL-1β). Linear regression analyses included lean body mass, appendicular lean mass and handgrip strength as dependent variables and the production capacity of individual cytokines or their composite scores as independent variables. All analyses were adjusted for age, height and body fat mass. Normalization cytokine production capacity values for monocyte count was performed by dividing the amount of produced cytokine in pg/L by the number of monocytes x 109/L. Then the cytokine production levels normalized for monocyte count were naturally log transformed due to skewness. To evaluate the influence of the capacity to stimulate M1 monocyte response upon stimulation with LPS and the secretory capacity of M1 monocytes upon stimulation with LPS together, for each subject the overall mean of the composite scores of the capacity to stimulate M1 monocyte response upon stimulation with LPS and the secretory capacity of M1 monocytes upon stimulation with

LPS were calculated. All analyses were performed for males and females separately and were adjusted for age, height and body fat mass. *P*-values < 0.05 were regarded as statistically significant.

6.3 Results

Table 6.1 lists the characteristics of the included 386 participants of The Leiden Longevity Study, a study consisting of offspring from long-lived Caucasian siblings and the partners thereof. As some innate immune characteristics have been shown to be sex specific, data were analysed stratified for men and women. Mean age was 64.2 years (SD 6.5) for men and 61.9 years (SD 6.4) for women. In men, mean lean body mass, mean appendicular lean mass and mean handgrip strength were higher compared to women. Median IFN-γ production capacity upon stimulation with LPS was higher in men, and median GM-CSF production capacity upon stimulation with LPS was higher in women. Median IL-6, TNF-α, IL-12 and IL-1β production capacity upon stimulation with LPS were all higher in men compared to women. Data on IFN-γ and GM-CSF production capacity upon stimulation with Pam3Cys-SK4 were not available because of values below the detection limit. Cytokine production capacity upon stimulation with Pam3Cys-SK4 was not different between men and women.

6.3.1 Cytokine production capacity upon stimulation with LPS

Table 6.2 shows the association of production capacity levels of cytokines that stimulate M1 monocyte response upon stimulation with LPS and production capacity levels of cytokines that are secreted by M1 monocytes upon stimulation with LPS with lean body mass, appendicular lean mass and handgrip strength. In men, a higher IFN-γ and GM-CSF production capacity was significantly associated with higher lean body mass, higher appendicular lean mass and higher handgrip strength. Furthermore, a composite score was calculated from the standardized values of the production capacity levels of cytokines that stimulate M1 monocyte response (IFN-γ and GM-CSF). This composite score was significantly positively associated with lean body mass ($p = 0.014$), appendicular lean mass ($p = 0.018$) and handgrip strength ($p = 0.018$) in men. From the cytokines known to be secreted by M1 monocytes, a higher TNF-α production capacity was significantly positively associated with higher lean body mass ($p = 0.006$), higher appendicular lean mass

(p = 0.027) and higher handgrip strength (p = 0.020) in men. A higher IL-12 production capacity was also significantly associated with higher handgrip strength (p = 0.007), but not with lean body mass (p = 0.21) and appendicular lean mass (p = 0.47). The composite score made up from the production capacity levels of cytokines that are secreted by M1 monocytes (IL-6, TNF-α, IL-12 and IL-1β) was borderline significantly associated with lean body mass (p = 0.059) and handgrip strength (p = 0.09) in men. In women, no significant associations were found. Additional adjustment for comorbidities, family trait for longevity and physical activity did not materially change the results (data not shown). Also normalizing cytokine production levels for monocyte count and adjustment for monocyte concentration did not materially change the results (data not shown).

Next, subjects were categorized into quartiles of overall inflammatory capacity of M1 monocytes depending on the overall mean of the composite scores of the production capacity levels of cytokines that stimulate M1 monocyte response and the production capacity levels of cytokines that are secreted by M1 monocytes. Figure 6.2 shows that in men, higher overall inflammatory capacity of M1 monocytes upon stimulation with LPS was associated with higher lean body mass (p for trend = 0.013), higher appendicular lean mass (p for trend = 0.055) and higher handgrip strength (p for trend = 0.015). Men in the lowest overall inflammatory capacity of M1 monocytes quartile (n = 46) had on average 59.1 kg (SE 0.6) lean body mass, 26.7 kg (SE 0.2) appendicular lean mass and 47.0 kg (SE 1.0) handgrip strength. Men in the highest overall inflammatory capacity of M1 monocytes quartile (n = 46) had on average 61.2 kg (SE 0.6) lean body mass, 27.5 kg (SE 0.2) appendicular lean mass and 50.1 kg (SE 1.0) handgrip strength. In women, no significant associations were found. Additional adjustment for comorbidities, family trait for longevity and physical activity did not materially change the results (data not shown). Also normalizing cytokine production levels for monocyte count and adjustment for monocyte concentration did not materially change the results (data not shown).

6.3.2 Cytokine production capacity upon stimulation with Pam-3Cys-SK4

Table 6.3 shows the relation between production capacity of cytokines that are secreted by monocytes upon Pam3Cys-SK4 stimulation and lean body mass, appendicular lean mass and handgrip strength in men and women. In men, no significant associations were found. In women, a higher IL-

6, TNF-α and IL-1β production capacity was significantly associated with a higher lean body mass and higher appendicular lean mass, but not with handgrip strength. A higher IL-12 production capacity was in women significantly associated with a higher lean body mass. The composite score calculated from the production capacity levels of cytokines that are secreted by M1 monocytes (IL-6, TNF-α, IL-12 and IL-1β) was significantly associated with lean body mass (p = 0.022) and appendicular lean mass (p = 0.028) in women, but not in men. Additional adjustment for comorbidities, family trait for longevity and physical activity did not change the results significantly (data not shown). Normalizing the cytokine production levels for monocyte count together with an adjustment for monocyte concentration did also not materially change the results (data not shown).

6.4 Discussion

The aim of this study was to investigate whether the pro-inflammatory capacity of classically activated (M1) monocytes relates to muscle mass and strength in humans. We have shown that in men IFN-γ and GM-CSF production capacity as well as TNF-α production capacity upon stimulation with LPS were positively associated with lean body mass, appendicular lean mass and handgrip strength. IL-12 production capacity upon stimulation with LPS was also positively associated with handgrip strength. In addition, male subjects with a higher overall inflammatory capacity of M1 monocytes upon stimulation with LPS had higher lean body mass and higher handgrip strength. In women no association between inflammatory capacity of M1 monocytes upon stimulation with LPS and muscle characteristics were found. Upon stimulation with Pam3Cys-SK4, IL-6, TNF-α and IL-1β were positively associated with lean body mass and appendicular lean mass in women, but not in men. In women, IL-12 cytokine production capacity upon stimulation with Pam3Cys-SK4 was also positively associated with lean body mass.

Inflammatory capacity of M1 monocytes was measured by stimulating whole blood ex vivo with LPS or Pam3Cys-SK4, both M1 phenotype activators in a highly standardized way (Mills *et al.*, 2000; Wang *et al.*, 2007; Navarro-Xavier *et al.*, 2010). Earlier studies showed that the intra-individual variation of cytokine production upon stimulation with LPS was over 50% of the inter-individual day-to-day variation (Van der Linden *et al.*, 1998; Damsgaard *et al.*, 2009a). Furthermore, over 50% of this intra-individual variation is known to be genetically determined (De Craen *et al.*, 2005). Whole blood

stimulated cytokine production is known to be well correlated with isolated monocytic cytokine production (Damsgaard *et al.*, 2009a). However, also other immune cells than monocytes produce cytokines upon stimulation and could therefore have had a contribution to the total amount of produced cytokines (Cassatella, 1995). In fact, cytokine production upon stimulation in whole blood reflects the complex interaction between different cell types in vivo. Neutrophil granulocytes migrate into the tissue after injury before the migration of monocytes, are involved in skeletal muscle repair and are stimulated by GM-CSF (Teixeira *et al.*, 2003). We assume that the capacity to produced cytokines in general is most clinically relevant for the skeletal muscle. We found that normalizing and adjusting the pro-inflammatory capacity of M1 monocytes for monocyte count did not change the results. This suggests that a high cytokine production capacity is not the result of a high white monocyte count per se, but a functional characteristic of monocytes themselves.

In mice, monocytes with a pro-inflammatory phenotype like M1 monocytes are known to respond to muscle injury by migrating from the blood into the muscle tissue, clearing necrotic tissue and stimulating the muscle's progenitor cells (Arnold *et al.*, 2007). We assume that the capacity to respond to LPS or Pam3Cys-SK4 is a proxy for the capacity to respond to stimuli from the skeletal muscle like muscle injury. In the present study, we measured the capacity to stimulate M1 monocyte response as well as the secretory capacity of M1 monocytes. The cytokines that stimulate M1 monocyte response are mainly produced by immune cells in the whole blood other than monocytes and induce not only cytokine production response (Kamijo *et al.*, 1993; Bundschuh *et al.*, 1997), but also the phagocytic capacity of M1 monocytes (Mur *et al.*, 1987). The stimulatory capacity of M1 monocytes upon stimulation with LPS was positively associated with lean body mass, appendicular lean mass and handgrip strength in men. This suggests that stimulation of M1 monocytes is important for the function of M1 macrophages within the muscle. It is known that IFN-γ has a "priming" function on monocytes by shifting the dose response curve of monocytes such that the threshold for LPS to affect gene regulation in monocytes is lower (Schoenmaker *et al.*, 2006). IFN-γ, as well as GM-CSF, cause a more sensitive and heightened response to LPS (Kamijo *et al.*, 1993; Bundschuh *et al.*, 1997) and assumingly also to stimuli from the skeletal muscle like muscle injury. In line with this explanation, we report a positive association between handgrip strength and the capacity of M1 monocytes to secrete IL-12 upon LPS stimulation, a cytokine which is known to induce T- cells and NK cells to produce IFN-γ, GM-CSF and TNF-α

(Trinchieri, 2003).

We observed in men a positive relation between the secretory production capacity of TNF-α by M1 monocytes upon stimulation LPS and lean body mass, appendicular lean mass and handgrip strength. In women, we observed a positive relation between the secretory production capacity of TNF-α by M1 monocytes upon stimulation Pam3Cys-SK4 and lean body mass and appendicular lean mass. TNF-α is an extensively studied cytokine with pleiotropic effects. The physiological effect of TNF-α is to regulate myogenesis and muscle regeneration through its activation of p38 MAPK (Warren *et al.*, 2002; Chen, Jin & Li, 2007). This effect is known to be independent of IL-6 (Warren *et al.*, 2002), which could explain the finding that IL-6 production capacity by M1 monocytes upon stimulation with LPS was not significantly associated with muscle mass and strength in our study. In pathologic conditions, like inflammatory myopathies, cachexia and age-related chronic systemic inflammation, TNF-α is related to muscle wasting (Morley, Thomas & Wilson, 2006; Salomonsson & Lundberg, 2006) and is capable to induce muscle proteolysis (Costelli *et al.*, 1993). However, in these pathological conditions TNF-α production is a chronic reaction cellular damage. During chronological aging, the level of systemically circulating cytokines increase, whereas the acutely produced cytokines by leukocytes in response to TLR stimulation or resistance exercise decrease (Van den Biggelaar *et al.*, 2004; Przybyla *et al.*, 2006).Furthermore, a high pro-inflammatory cytokine production capacity upon stimulation with LPS has been associated with low C-reactive proteins levels in subjects aged 85 years (Wijsman *et al.*, 2011). This suggests that cytokine production capacity upon stimulation with LPS should therefore be interpreted differently from levels of systemically circulating cytokines. Earlier we found in a selected group of subjects aged 85 years that the decline over time in handgrip strength, is higher in subjects with a higher TNF-α production capacity upon stimulation with LPS (Taekema *et al.*, 2007). A possible explanation is that TNF-α production capacity is protective for the skeletal muscle earlier in life, but has detrimental effects on age-related loss of muscle mass and strength later in life, as proposed by the theory of antagonistic pleiotropy (Williams, 1957; Van Bodegom *et al.*, 2007). Longitudinal studies in young subjects are needed to prove this explanation.

In contrast to men, no relation between pro-inflammatory capacity of M1 monoyctes upon stimulation with TLR-4 agonist (LPS) and muscle mass and muscle strength was found in women. In women however, we did find significant relations between pro-inflammatory capacity of M1 monocytes upon

stimulation with TLR-2/1 agonist (Pam3Cys-SK4) and muscle mass. These findings indicate that within the investigated relation the TLR-4 pathway is predominant in men and the TLR-2/1 pathway in predominant in women. A recent study in mice showed that coxsackievirus B3 infection causes in leukocytes and hearth muscle tissue an up-regulation of TLR-4 in males and up-regulation of TLR-2/1 in females (Roberts *et al.*, 2012). In humans it has been shown that monocytic TLR-2/1 expression, but not monocytic TLR-4 expression, was significantly higher in women compared to men (Ono *et al.*, 2005). We found that the cytokine production capacity in men compared to women was more prominent upon TLR-4 stimulation, than upon TLR-2/1 stimulation. Others found that TNF-α cytokine production capacity per monocyte was higher in men compared to women (Aulock *et al.*, 2006). However, they also found that this difference was similar when a TLR-2/1 instead of a TLR-4 stimulator was used. Nevertheless, changes in sex hormones during the menstrual cycle are known to influence the cytokine production upon TLR-4 stimulation differently in comparison to TLR-2/1 stimulation (Dennison, 2012). Future studies are needed to investigate sex differences in the TLR-4 and TLR-2/1 pathways.

Our findings are consistent with studies showing that inflammatory cells have a beneficial effect on skeletal muscle. Studies in mice have shown that depletion of monocytes is associated with a prolonged clearance of necrotic myofibers, a tendency for increased muscle fat accumulation, an impaired membrane lesion repair and satellite cell differentiation and prevention of muscle fiber growth after muscle injury (Summan *et al.*, 2006; Arnold *et al.*, 2007; Tidball & Wehling-Henricks, 2007). We are the first to report that a higher overall inflammatory capacity of M1 monocytes is associated with a higher lean body mass and higher handgrip strength in men. These results suggest that future anti-inflammatory therapies targeting the effects of inflammation mediated by the innate immune system on age-related diseases like sarcopenia and dynapenia may be potentially harmful for human skeletal muscle.

The strengths of this study include the relatively large number of subjects in which innate immune capacity was measured and the availability of measurements for muscle mass as well as muscle strength. A first limitation is the cross-sectional study design, which makes causal inference difficult. Another limitation is that the inflammatory capacity of M1 monocytes was measured using a ligand secreted by bacteria and not by injured human muscle. However, trauma and pathogens elicit the same inflammatory response and

TLR-4 and TLR-2/1, the pathogen recognition receptors (PRR) for LPS and Pam3Cys-SK4, respectively, are also known to recognize endogenous danger signals (Bianchi & Manfredi, 2009). Furthermore, in the present study we did not investigate the inflammatory capacity of M2 monocytes, since we stimulated whole blood with LPS and Pam3Cys-SK4 and not with IL-4, IL-13 or IL-10 (see Figure 6.1). Future studies are needed to investigate the relation between inflammatory capacity of M1 and M2 monocytes and the relation between the inflammatory capacity of monocytes with respect to that of macrophages.

In conclusion, within a middle aged human population we showed that the inflammatory capacity of M1 monocytes, measured by stimulating whole blood ex-vivo with LPS, is strongly positively associated with muscle mass and strength in men. In women, the inflammatory capacity of M1 monocytes, measured by stimulating whole blood ex-vivo with Pam3Cys-SK4, is strongly positively associated with muscle mass.

Table 6.1. Characteristics of Leiden Longevity Study including middle aged men and women.

Characteristic	Men N=191	Women N=195
Clinical characteristics		
Age, years	64.2 (6.5)	61.9 (6.4)
Comorbidity n, (%)		
Diabetes mellitus	12 (7.1)	8 (4.7)
Hypertension	38 (22.5)	50 (29.1)
Myocardial infarction	7 (4.1)	1 (0.6)
Stroke	7 (4.1)	4 (2.3)
History of malignancy	9 (5.3)	13 (7.6)
Rheumatoid arthritis	0	2 (1.2)
Family longevity trait n, (%)	111 (58.1)	83 (42.6)
Body composition		
Height, cm	177.8 (7.0)	165.4 (6.0)
Weight, kg	84.9 (11.4)	71.5 (12.3)
Body fat mass, kg	21.3 (7.4)	25.2 (9.1)
Lean body mass, kg	60.0 (6.9)	43.6 (4.8)
Appendicular lean mass, kg	27.0 (3.4)	18.9 (2.5)
Handgrip strength, kg	48.0 (8.1)	30.0 (5.5)
IPAQ vigorous activity, h/week (median, IQR)	0.3 (0.0-5.0)	0.0 (0.0-3.5)
Hematologic measurements		
Leukocyte concentration $*10^9$/L	6.43 (1.44)	6.34 (1.42)
Monocytes concentration $*10^9$/L	0.55 (0.20)	0.47 (0.14)
Cytokine production capacity upon stimulation with LPS		
Capacity to stimulate M1 monocyte response (median, IQR)		
IFN-γ, pg mL^{-1}	1877 (652-4757)	1766 (856-4097)
GM-CSF, pg mL^{-1}	118 (71-208)	154 (91-289)
Secretory capacity of M1 monocytes (median, IQR)		
IL- 6, pg mL^{-1}	94231 (78474-121669)	82783 (67488-102946)
TNF-α, pg mL^{-1}	9487 (6406-13861)	8091 (5805-11020)
IL-12, pg mL^{-1}	5003 (3899-6793)	4583 (3533-5941)
IL-1β, pg mL^{-1}	12184 (9496-16081)	10080 (7428-13145)
Cytokine production capacity upon stimulation with $Pam_3Cys\text{-}SK_4$		
Secretory capacity of M1 monocytes (median, IQR)		
IL- 6, pg mL^{-1}	5835 (1162-11440)	4605 (971-9959)
TNF-α, pg mL^{-1}	135 (37-365)	122 (33-314)
IL-12, pg mL^{-1}	463 (125-959)	367 (125-833)
IL-1β, pg mL^{-1}	66 (32-160)	55 (24-115)

Data are presented as mean (SD) unless stated otherwise. Family longevity trait: offspring of nonagenarian siblings. LPS: lipopolysaccharide. $Pam_3Cys\text{-}SK_4$: tripalmitoyl-S-glycerylcysteine, M1: classically activated. IFN: interferon. GM-CSF: granulocyte macrophage colony-stimulating factor. IL: interleukin. TNF: tumor necrosis factor. IQR: interquartile range.

Table 6.2. Production capacity of cytokines that stimulate M1 monocyte response upon stimulation with LPS or are secreted by M1 monocytes upon stimulation with LPS related to lean body mass, appendicular lean mass and handgrip strength in men and women.

	Lean body mass		Appendicular lean mass		Handgrip strength	
	kg (SE)	p	kg (SE)	p	kg (SE)	p
Men						
Capacity to stimulate M1 monocyte response						
IFN-γ	**0.78 (0.31)**	**0.013**	**0.30 (0.13)**	**0.025**	**1.35 (0.52)**	**0.018**
GM-CSF	**0.60 (0.29)**	**0.038**	**0.27 (0.12)**	**0.032**	**0.97 (0.48)**	**0.044**
Composite score	**0.79 (0.32)**	**0.014**	**0.33 (0.14)**	**0.018**	**1.28 (0.54)**	**0.018**
Secretory capacity of M1 monocytes						
IL-6	0.46 (0.30)	0.14	0.12 (0.13)	0.37	0.23 (0.50)	0.64
TNF-α	**0.78 (0.28)**	**0.006**	**0.27 (0.12)**	**0.027**	**1.10 (0.47)**	**0.020**
IL-12	0.35 (0.35)	0.21	0.09 (0.12)	0.47	**1.26 (0.46)**	**0.007**
IL-1β	0.19 (0.31)	0.53	0.04 (0.13)	0.76	0.22 (0.51)	0.66
Composite score	0.69 (0.36)	0.059	0.20 (0.16)	0.20	1.03 (0.60)	0.09
Women						
Capacity to stimulate M1 monocyte response						
IFN-γ	0.01 (0.23)	0.96	-0.07 (0.10)	0.53	-0.13 (0.38)	0.74
GM-CSF	0.34 (0.22)	0.12	0.14 (0.10)	0.15	-0.10 (0.36)	0.78
Composite score	0.21 (0.24)	0.39	0.04 (0.11)	0.70	-0.13 (0.40)	0.74
Secretory capacity of M1 monocytes						
IL-6	0.05 (0.25)	0.83	-0.01 (0.11)	0.90	0.20 (0.42)	0.64
TNF-α	-0.10 (0.23)	0.65	-0.10 (0.10)	0.34	-0.07 (0.38)	0.86
IL-12	-0.18 (0.24)	0.46	-0.11 (0.11)	0.32	0.34 (0.40)	0.40
IL-1β	0.18 (0.22)	0.41	0.06 (0.10)	0.53	0.27 (0.36)	0.46
Composite score	0.05 (0.28)	0.87	-0.03 (0.12)	0.84	0.30 (0.48)	0.53

Estimates are standardized per 1 SD increase in cytokine level. p: p-value. M1 monocyte: classically activated monocytes by LPS stimulation. IFN: interferon. GM-CSF: granulocyte macrophage colony-stimulating factor. IL: interleukin. TNF: tumor necrosis factor. SE= standard error. Cytokine values were natural log transformed. Capacity to stimulate M1 monocyte response composite score: mean Z-score of IFN-γ and GM-CSF values. Secretory capacity of M1 monocytes composite score: mean Z-score of IL-6, TNF-α, IL-12 and IL-1β values. Results were adjusted for age, height and body fat mass. Values in bold are statistically significant (P<0.05).

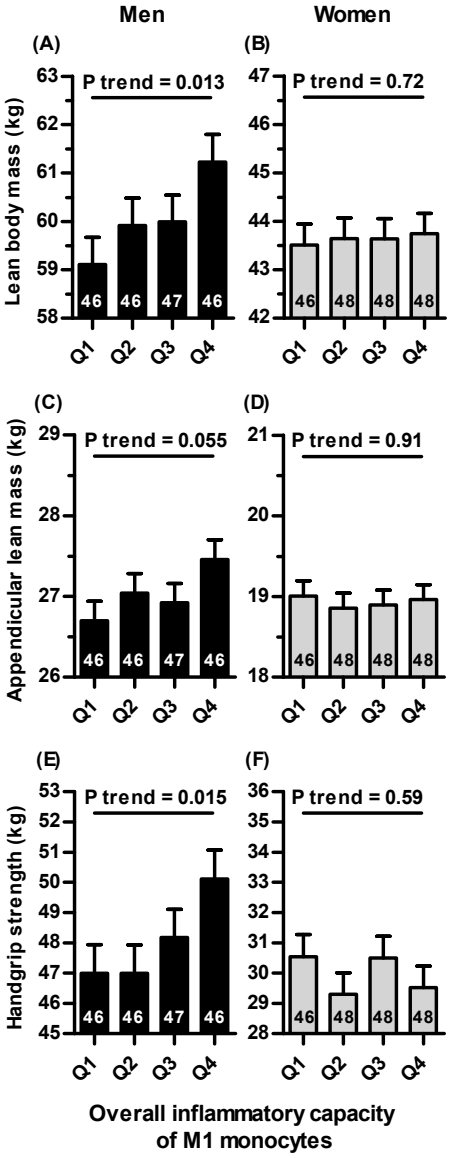

Figure 6.2. Overall inflammatory capacity of M1 monocytes upon stimulation with LPS in men (A,C,E) and women (B,D,F) related to lean body mass (A,B), appendicular lean mass (C,D) and handgrip strength (E,F). Bars represent quartiles of mean overall inflammatory capacity of M1 monocytes upon stimulation with LPS (SE) adjusted for age, height and body fat mass. Overall inflammatory capacity of M1 monocytes was defined as overall mean of the composite scores of the capacity to stimulate M1 response and the secretory capacity of M1 monocytes upon stimulation with LPS. M1: classically activated. Kg: kilogram. Numbers in bars indicate the number of subjects. Data and *p*-for trends are calculated using linear regression with adjustment for age, height and body fat mass. Values in bold are statistically significant ($P < 0.05$).

Table 6.3. Production capacity of cytokines that are secreted by M1 monocytes upon stimulation with Pam3Cys-SK4 related to lean body mass, appendicular lean mass and handgrip strength in men and women.

	Lean body mass		Appendicular lean mass		Handgrip strength	
	kg (SE)	p	kg (SE)	p	kg (SE)	p
Men						
Secretory capacity of M1 monocytes						
IL-6	0.26 (0.29)	0.36	0.10 (0.12)	0.40	-0.10 (0.47)	0.83
TNF-α	0.40 (029)	0.17	0.14 (0.13)	0.26	0.04 (0.49)	0.93
IL-12	0.35 (0.29)	0.23	0.11 (0.13)	0.37	0.16 (0.49)	0.75
IL-1β	0.10 (0.32)	0.75	0.03 (0.14)	0.85	-0.03 (0.52)	0.53
Composite score	0.36 (0.32)	0.26	0.14 (0.14)	0.97	-0.02 (0.54)	0.97
Women						
Secretory capacity of M1 monocytes						
IL-6	**0.53 (0.22)**	**0.016**	**0.20 (0.10)**	**0.038**	0.41 (0.36)	0.25
TNF-α	**0.49 (0.22)**	**0.027**	**0.21 (0.10)**	**0.038**	0.21 (0.37)	0.57
IL-12	**0.43 (0.21)**	**0.044**	0.18 (0.10)	0.065	0.29 (0.35)	0.41
IL-1β	**0.68 (0.26)**	**0.009**	**0.31 (0.11)**	**0.008**	0.29 (0.43)	0.49
Composite score	**0.59 (0.25)**	**0.022**	**0.25 (0.11)**	**0.028**	0.25 (0.42)	0.54

Estimates are standardized per 1 SD increase in cytokine level. p: p-value. M1 monocyte: classically activated monocytes by $Pam_3Cys\text{-}SK_4$ stimulation. IL: interleukin. TNF: tumor necrosis factor. SE= standard error. Cytokine values were natural log transformed. Secretory capacity of M1 monocytes composite score: mean Z-score of IL-6, TNF-α, IL-12 and IL-1β values. Results were adjusted for age, height and body fat mass. Values in bold are statistically significant (P<0.05).

Chapter 7

Variants of the IL-10 gene determine muscle strength in elderly from rural Africa: a candidate gene study

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Abstract

Background: Recently, it has been shown that the capacity of the innate immune system to produce cytokines relates to skeletal muscle mass and strength in older persons. The interleukin-10 (IL-10) gene regulates the production capacities of IL-10 and tumour necrosis factor-α (TNF-α). In rural Ghana, IL-10 gene variants associated with different production capacities of IL-10 and TNF-α are enriched compared with Caucasian populations. In this setting, we explored the association between these gene variants and muscle strength.
Methods: Among 554 Ghanaians aged 50 years and older, we determined 20 single nucleotide polymorphisms in the IL-10 gene, production capacities of IL-10 and TNF-α in whole blood upon stimulation with lipopolysaccharide (LPS), and handgrip strength as a proxy for skeletal muscle strength. We distinguished pro-inflammatory haplotypes associated with low IL-10 production capacity and anti-inflammatory haplotypes with high IL-10 production capacity.
Results: We found that distinct haplotypes of the IL-10 gene associated with handgrip strength. A pro-inflammatory haplotype with a population frequency of 43.2% was associated with higher handgrip strength ($p = 0.015$). An anti-inflammatory haplotype with a population frequency of 7.9% was associated with lower handgrip strength ($p = 0.006$).
Conclusions: Variants of the IL-10 gene contributing to a pro-inflammatory cytokine response associate with higher muscle strength, whereas those with anti-inflammatory response associate with lower muscle strength. Future research needs to elucidate whether these effects of variation in the IL-10 gene are exerted directly through its role in the repair of muscle tissue or indirectly through its role in the defence against infectious diseases.

7.1 Introduction

Interleukin-10 (IL-10) is an anti-inflammatory cytokine with important regulatory effects on inflammatory responses. It down-regulates the antigen presenting function and inhibits the production of pro-inflammatory cytokines like tumour necrosis factor α (TNF-α) by various immune cells (Hofmann *et al.*, 2012). In mice, immune cells producing cytokines are crucial for the repair of skeletal muscle tissue (Arnold *et al.*, 2007; Bencze *et al.*, 2012; Deng *et al.*, 2012; Gao *et al.*, 2012). We have recently shown that a higher TNF-α

production capacity of immune cells is positively related to muscle mass and muscle strength in a middle-aged Dutch population (Beenakker *et al.*, 2013).

The capacity to produce IL-10 and TNF-α upon whole-blood stimulation with lipopolysaccharide (LPS) varies between individuals. This variation is for more than 50% attributable to genetic determinants (Westendorp *et al.*, 1997; De Craen *et al.*, 2005; Damsgaard *et al.*, 2009a). The IL-10 gene is highly polymorphic (Eskdale *et al.*, 1998; Kube *et al.*, 2001; Kuningas *et al.*, 2009) and its haplotypes are transcribed differently (Kurreeman *et al.*, 2004). This inter-individual variation is extended by variation in the IL-10 haplotype structure and distribution between ethnicities (Eskdale *et al.*, 1998; Moraes *et al.*, 2003). We have earlier reported that specific IL-10 gene variants are enriched in Ghanaian elderly living under adverse conditions (Kuningas *et al.*, 2009). These variants have functional significance: some are related to a pro-inflammatory cytokine production capacity, with lower IL-10 and higher TNF-α levels upon whole-blood stimulation with LPS, while others are related to an inversed anti-inflammatory response (Kuningas *et al.*, 2009; May *et al.*, 2009b; Boef *et al.*, 2012). Interestingly, this IL-10 haplotype structure is less present in Caucasian populations living under affluent conditions (Moraes *et al.*, 2003; Kuningas *et al.*, 2009), possibly because balanced selection has conserved this haplotype structure in populations under adverse conditions. The functional variation in the genetic determinants of cytokine production capacity forms a meaningful instrument to study the effects of different cytokine production capacities largely free from confounders and reverse causality (Davey Smith & Ebrahim, 2003).

For the present study, we had the unique opportunity to study handgrip strength of individuals aged 50 years and older in the Ghanaian population of which we have extensively characterised the IL-10 gene variants and their effects on cytokine production capacity (Kuningas *et al.*, 2009; May *et al.*, 2009b; Boef *et al.*, 2012). This study aims to investigate the relation between the pro- and anti-inflammatory IL-10 gene variants, that are not present in Caucasian populations, and handgrip strength as a proxy of overall muscle strength. To account for the possible effects of ill health on handgrip strength, the analyses were also performed after exclusion of subjects with underweight.

7.2 Methods

7.2.1 Research area

This study was performed in a remote, rural, and underdeveloped area in the Upper East Region in Ghana in West Africa. The vast majority of the inhabitants are involved in non-commercial agriculture performed by manual labour. Infectious diseases are the main causes of death (Ghana Health Service, 2005). The prevalence of human immunodeficiency virus (HIV) is low (< 4%) compared with other sub-Saharan regions (Ghana Health Service, 2005). Since 2002, we have followed a horticultural population in the Garu-Tempane District in the Upper East Region, comprising approximately 25,000 inhabitants living in circa 40 villages. For each household, we determined the household property value and the socioeconomic status in 2007 according to the Demographic and Health Survey method (Van Bodegom *et al.*, 2009). Elaborate descriptions of the research population have been given elsewhere (Meij, van Bodegom & Laar, 2007; Kuningas *et al.*, 2009; Van Bodegom *et al.*, 2009; Koopman *et al.*, 2012).

7.2.2 Ethical approval

Ethical approval was given by the Ethical Review Committee of Ghana Health Services, the Committee Medical Ethics of the Leiden University Medical Center, and by the local chiefs and elders. Because of illiteracy, informed consent was obtained orally from the participants in the local language. A consent form was read out to each participant with an explanation on the purpose and conduction of this research project.

7.2.3 DNA collection and genotyping

We collected buccal swabs between 2002 and 2006 for 4336 individuals (Kuningas *et al.*, 2009). Common genetic variation (minor allele frequency ≥ 5%) in the IL-10 gene region was determined by genotyping 20 SNPs, selected from the Yoruba population in the HapMap database (release #21, $r^2 = 0.8$) and genotyped using mass spectrometry (Seqeunom Inc, San Diego, USA). All SNPs were in Hardy-Weinberg equilibrium, with one exception where a minor deviation was observed (Kuningas *et al.*, 2009). We have recently reported that population stratification is unlikely to influence associations with genetic variation in autosomal genes, since analysis of au-

tosomal DNA, mtDNA, and y-chromosomal genetic variation patterns in the research area revealed that female-mediated gene flow is nearly fully random, whereas male-mediated gene flow is highly reduced (Sanchez-Faddeev *et al.*, 2013). This genetic substructure is an immediate result of the patrilocal society. We addressed residual population stratification by adjusting all analyses for tribe. Familial relatedness among individuals was addressed by adjusting all analyses for household.

7.2.4 Handgrip strength and BMI

Handgrip strength and BMI were measured in 2009 and 2010 in 923 individuals aged 50 years and older, recruited independently from the genetic samples. Data on the IL-10 gene were available for 554 of them. We consecutively visited all villages in the research area, in which we set up a mobile fieldwork station. We approached all individuals aged 50 years and older and brought less mobile participants by car. Inclusion was limited by the duration of both field visits. Individuals did not participate if they were unable to leave the house, were absent from the research area for a longer period, or refused participation. Handgrip strength was measured using a calibrated Jamar hand dynamometer (Sammons Preston Inc., Bolingbrook, IL) with the participant in an upright position and the arm of the measured hand unsupported parallel to the body. The width of the dynamometer's handle was adjusted to each participant's hand size so that the middle phalanges rested on the inner handle. The participants were instructed to exert maximal force once by each hand. We used the highest measurement of both hands in our analyses. Body height and weight were measured by a calibrated length scale and weighing scale. A BMI of 18.5 kg/m^2 or lower was defined as underweight and a BMI above 18.5 kg/m^2 as a normal BMI, according to the classification of the Food and Agriculture Organization and World Health Organization (Shetty & James, 1994; World Health Organization, 2003).

7.2.5 Cytokine production capacity

The production capacities of IL-10 and TNF-α were measured in blood samples that were taken in 2005, 2006, or 2008. Previous publications, studying measurements from these years separately, have reported the procedure by which the blood samples were processed (May *et al.*, 2009a,b; Boef *et al.*, 2012). Venous blood was locally incubated with E. coli LPS, the supernatants frozen and shipped for measurement of cytokine concentrations in

the Netherlands by enzyme-linked immunosorbent assay (ELISA). The procedure has been reported to have a small intra-individual compared with the inter-individual variation Damsgaard *et al.* (2009a) and to be replicable with an interval of two years in this research area (May *et al.*, 2009a; Boef *et al.*, 2012). We combined the measurements from all three years of 1177 individuals of whom data on the IL-10 gene were also available.

7.2.6 Statistical analyses

The program Haploview (Barrett *et al.*, 2005) was used to test for Hardy-Weinberg equilibrium. Statistical analyses were performed with IBM SPSS Statistics version 20 and StataCorp Stata/SE version 12.0. The relations between IL-10 gene SNPs and haplotype copies, cytokine production capacities, and handgrip strength were assessed by linear mixed models. These analyses were adjusted for age, sex, and tribe as fixed factors and household as a random factor. Analyses with handgrip strength were additionally adjusted for height as a fixed factor. Cytokine concentrations were natural-logarithmically transformed due to skewedness and standardised as z-scores ((individual level-mean level)/standard deviation) per sex (Aulock *et al.*, 2006) within each year of measurement. The z-scores of 2005, 2006, and 2008 were averaged into one z-score per individual. Haplotypes were defined as pro-inflammatory if associated with lower levels of IL-10 and higher levels of TNF-α upon stimulation with LPS. Haplotypes were defined as anti-inflammatory if associated with higher levels of IL-10 and lower levels of TNF-α upon stimulation with LPS (Kuningas *et al.*, 2009). In all haplotype analyses the posterior probabilities of pairs of haplotypes per individual, as estimated by PHASE (Stephens, Smith & Donnelly, 2001), were used as weights.

7.3 Results

Table 7.1 displays the characteristics of 554 individuals aged 50 years and older of whom IL-10 gene variants and handgrip strength were known. Their characteristics were similar as compared with all 4336 individuals of whom IL-10 gene variants were measured and with all 923 individuals of whom handgrip strength was measured (data not shown). Approximately half of them had a body mass index (BMI) of 18.5 kg/m^2 or lower, which is regarded as underweight (Shetty & James, 1994; World Health Organization, 2003). Mean handgrip strength was 27.3 kg (SD 7.6) for those with a normal BMI and

Table 7.1. Characteristics of the study sample *n* = 554.

Characteristic	Women (*n* = 358)	Men (*n* = 196)
Age, years	63.4 (9.2)	73.0 (9.2)
Tribe, *n* (%)		
Bimoba	252 (70.4)	142 (72.4)
Kusasi	84 (23.5)	44 (22.4)
Mamprusi	12 (3.4)	2 (1.0)
Fulani	1 (0.3)	2 (1.0)
Busanga	8 (2.2)	2 (1.0)
Other or unknown	1 (0.3)	4 (2.0)
Number of households, *n*	299	190
Household property value in US$, median (IQR)	1183 (585-2055)	1028 (580-1782)
Clinical measurements		
Height, cm	157.9 (6.7)	166.0 (6.8)
Weight, kg	45.4 (7.5)	49.4 (7.8)
Body mass index, kg/m^2	18.2 (2.5)	17.9 (2.3)
Body mass index ≤ 18.5 kg/m^2, *n* (%)	204 (57.0)	113 (57.6)
Handgrip strength, kg	23.4 (5.9)	29.2 (8.1)

Data are presented as means with standard deviations unless otherwise specified.

IQR: interquartile range.

24.1 kg (SD 6.7) for those with underweight. Table 7.2 shows that handgrip strength did not differ between tribes.

7.3.1 IL-10 gene variants and cytokine production capacity

It has been previously shown that several single nucleotide polymorphisms (SNPs) in the IL-10 gene region influence the production capacities of IL-10 and TNF-α measured in two independent groups in 2006 and 2008 in this research area (Kuningas *et al.*, 2009; Boef *et al.*, 2012). First we confirmed that these relations were present in 1177 individuals of whom IL-10 gene variants were known and of whom measurements of cytokine production capacities were combined from 2005, 2006, and 2008 (Figure 7.1A). When restricting this group to the individuals of whom handgrip strength was also known (*n* = 457), a similar pattern was present (Figure 7.1B).

It has been previously shown that the SNPs in the IL-10 gene region constitute two haplotypes that influence the production capacities of IL-10 and

Table 7.2. Association between tribe and handgrip strength.

	Bimoba (*n* = 394)	Kusasi (*n* = 128)	Mamprusi (*n* = 14)	Fulani (*n* = 3)	Busanga (*n* = 10)	Other (*n* = 3)	*p*
Handgrip strength, kg	26.6 (0.3)	26.8 (0.6)	26.3 (1.7)	21.9 (3.6)	30.7 (2.0)	27.7 (2.8)	0.30

Handgrip strength is given as mean with standard error. Values have been adjusted for age and sex. Difference between the tribes has been tested by ANCOVA.

TNF-α: a pro-inflammatory haplotype 1 and an anti-inflammatory haplotype 3 (Kuningas *et al.*, 2009; Boef *et al.*, 2012). We reanalysed the relation between the haplotypes of the IL-10 gene and cytokine production capacities in the 1177 individuals of whom IL-10 gene variants were known and of whom measurements of cytokine production capacities were combined from 2005, 2006, and 2008. We confirmed an additive genetic effect for both haplotypes. With each additional copy of the pro-inflammatory haplotype 1, z-scores of IL-10 production capacity were 0.08 lower (SE 0.04; p for trend = 0.028) and z-scores of TNF-α production capacity were 0.11 higher (SE 0.04; p for trend = 0.001). With each additional copy of the anti-inflammatory haplotype 3, z-scores of IL-10 production capacity were 0.19 higher (SE 0.07; p for trend = 0.005) and z-scores of TNF-α were 0.10 lower (SE 0.07; p for trend = 0.167). When restricting this group to the individuals of whom handgrip strength was also known (n = 457), similar patterns were found (data not shown). Haplotype 2 was not related with the production capacities of IL-10 (p for trend = 0.813) or TNF-α (p for trend = 0.364) and was used in this study as a negative control. Results were not different between men and women (data not shown).

7.3.2 IL-10 gene variants and handgrip strength

Figure 7.2A shows the twenty genotyped SNPs in the IL-10 gene region. Most of the SNPs tag a single linkage disequilibrium (LD) block. The haplotype structure and the frequencies of these haplotypes were previously calculated for all individuals of whom IL-10 gene variants were measured (n = 4336) (Kuningas *et al.*, 2009). Haplotype frequencies and Hardy-Weinberg equilibriums of the SNPs were not materially different when restricting this group to the individuals of whom handgrip strength was also known (Tables 7.3 and 7.4). Furthermore, allelic frequencies were not different between tribes, with a few exceptions between small and large tribes

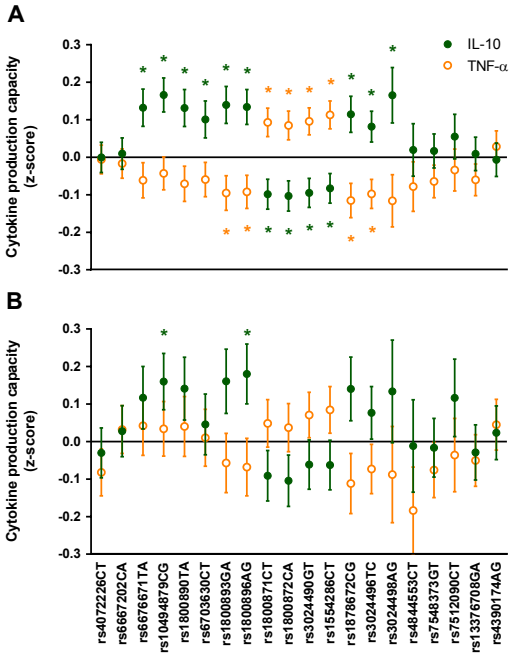

Figure 7.1. Association of IL-10 gene SNPs with cytokine production capacities. The relation between the minor allele of each IL-10 gene SNP and the production capacities of IL-10 and TNF-α for (A) individuals of whom IL-10 gene variants and cytokine production capacities were known (n = 1177) and (B) individuals of whom IL-10 gene variants, cytokine production capacities, and handgrip strength were known (n = 457). Cytokine production capacities are expressed as z-scores with standard error bars for carriers of at least one copy of the minor allele, adjusted for age, sex, tribe, and household (* $p < 0.05$).

(Table 7.5).

Figure 7.2B shows that in the individuals of whom IL-10 gene variants and handgrip strength were known carriers of distinct SNPs had higher or lower handgrip strength when compared with non-carriers. The pattern followed the predefined anti-inflammatory and pro-inflammatory haplotype structures shown in Figure 7.2A and Figure 7.1. Results were not different between men and women (data not shown).

Figure 7.3 shows handgrip strength for carriers and non-carriers of the pro-inflammatory haplotype 1 and the anti-inflammatory haplotype 3. We observed an additive genetic effect on handgrip strength, which increased with

Table 7.3. Haplotype frequencies in the study population and in the entire genotyped population.

	Haplotype frequencies	
	Study population	Entire genotyped population
Haplotype	(n = 554)	(n = 4336)
H1	0.457	0.432
H2	0.066	0.080
H3	0.063	0.079
H4	0.069	0.067
H5	0.048	0.053
H6	0.040	0.040
H7	0.048	0.035
H8	0.021	0.030
H9	0.026	0.022
H10	0.021	0.022
H11	0.012	0.016
H12	0.015	0.014

Differences in haplotype frequencies between both populations have been tested by the Pearson chi-squared test (p = 0.79).

each additional copy of the pro-inflammatory haplotype 1 and decreased with each additional copy of the anti-inflammatory haplotype 3. Among subjects with a normal BMI, the positive association between the pro-inflammatory haplotype 1 and handgrip strength was equally strong (p for interaction = 0.398) and the negative association between the anti-inflammatory haplotype 3 and handgrip strength was stronger (p for interaction = 0.009). Both haplotypes were not associated with BMI, neither in all subjects nor in those with BMI above 18.5 kg/m^2 (p for trend > 0.200). Contrary to haplotypes 1 and 3, haplotype 2 was not associated with handgrip strength (p for trend = 0.922). Results were not different between men and women (data not shown).

7.3.3 Cytokine production capacity and handgrip strength

Figure 7.4 shows that IL-10 and TNF-α production capacities were not related to handgrip strength, although an increase in IL-10 production capacity concurred with a declining trend in handgrip strength among subjects

with a normal BMI. When stratifying by sex, IL-10 production capacity was not associated with handgrip strength in either men or women (p for trend > 0.800), while TNF-α production capacity was positively associated with handgrip strength in men ($n = 128$; p for trend $= 0.007$) but not in women ($n = 329$; p for trend $= 0.819$).

7.4 Discussion

We found that distinct haplotypes of the IL-10 gene, associated with variation in the cytokine production capacities of immune cells, were related to handgrip strength in rural Ghana. A pro-inflammatory haplotype with a population frequency of 43.2% was associated with higher handgrip strength, while an anti-inflammatory haplotype with a population frequency of 7.9% was associated with lower handgrip strength. These associations were most outspoken after exclusion of individuals with underweight.

We investigated the effect of IL-10 gene variants as genetic determinants of the ex vivo production capacities of IL-10 and TNF-α by various immune cells in blood (Kuningas *et al.*, 2009; Boef *et al.*, 2012). The impact of genetic variants on the cytokine production capacity by specific immune cells is not precisely known, but whole-blood stimulation with LPS has been shown to particularly reflect the variance in cytokine production by monocytes (Damsgaard *et al.*, 2009a). By using genetic determinants, the analyses of the influence of cytokine production capacity on muscle strength are largely free from confounders and reverse causality (Davey Smith & Ebrahim, 2003).This is an advantage compared with our earlier research in which cytokine production capacity was only measured on the phenotypic level (Beenakker *et al.*, 2013).

Mice studies have shown that repair and maintenance of skeletal muscle tissue are dependent on the innate immune system (Arnold *et al.*, 2007; Bencze *et al.*, 2012; Deng *et al.*, 2012; Gao *et al.*, 2012). Critical for this role of the innate immune system are monocytes infiltrating muscle tissue after injury (Lu *et al.*, 2011b; Nguyen, Cheng & Koh, 2011) and producing pro-inflammatory cytokines, of which most notably TNF-α (Warren *et al.*, 2002; Chen *et al.*, 2005). As a counterbalance, the anti-inflammatory cytokine IL-10 down-regulates the pro-inflammatory functioning of monocytes and the production of pro-inflammatory cytokines such as TNF-α (Mosser & Zhang, 2008) and is associated with deceleration of skeletal muscle regeneration (Gao *et al.*, 2012). In humans, we have recently reported that a higher ca-

pacity to produce pro-inflammatory cytokines, including TNF-α, coexists with higher muscle strength and muscle mass (Beenakker *et al.*, 2013). We now show that the same associations exist between genetic determinants of the cytokine production capacity and handgrip strength. These findings support that cytokine production capacity might be important for human muscle repair and maintenance as well.

As an alternative explanation of the association between the IL-10 gene variants and muscle strength, pro-inflammatory IL-10 gene variants might yield a better resistance to infectious diseases and thereby a better resistance to muscle wasting due to disease. Cytokine production capacity is related to the incidence and severity of infectious diseases (Mege *et al.*, 2006). In rural Ghana, infectious diseases are the main causes of death (Ghana Health Service, 2005) and we have earlier observed in this research area that carriers of a pro-inflammatory IL-10 gene haplotype have a survival advantage when drinking from pathogen-rich sources like open wells and rivers (Kuningas *et al.*, 2009). Others have found another anti-inflammatory genetic variant associated with a higher IL-10 production capacity to be more prevalent among tuberculosis patients compared with healthy controls in Gambia (Awomoyi *et al.*, 2002). Such a mechanism could explain why no relation between a SNP on the IL-10 gene and handgrip strength was found in a Caucasian population living in an environment where the pathogenic burden is relatively low (Dato *et al.*, 2010).

Infectious diseases and malnutrition, which are common in this research area (Ghana Health Service, 2005; Hesselberg & Yaro, 2006; Ghana Statistical Service, 2009; Koopman *et al.*, 2012; Boef *et al.*, 2013), are closely associated with underweight (Shetty & James, 1994). In an attempt to account for the possible effects of ill health on handgrip strength, we repeated the analyses after exclusion of subjects with underweight. Among subjects with a normal BMI, we found an equal relation between the pro-inflammatory haplotype and a stronger relation between the anti-inflammatory haplotype and muscle strength. Moreover, we found that the haplotypes that were associated with handgrip strength were not associated with BMI. These findings indicate that the relation between IL-10 gene variants and handgrip strength is unlikely to be largely shaped by differences in health.

Although IL-10 gene variants were related to handgrip strength, the production capacity of IL-10 was not associated with handgrip strength. As a possible explanation, monocytes activated by a pro-inflammatory stimulus like LPS, migrate into injured muscle tissue and change only after two

days into macrophages with an anti-inflammatory phenotype (Arnold *et al.*, 2007). We measured the cytokine production capacity of IL-10 24 hours after stimulation with LPS, which could have been too early to measure the maximum IL-10 production capacity. In addition, IL-10 is known to have auto-regulatory effects, since it strongly inhibits IL-10 mRNA synthesis in LPS-activated monocytes (De Waal Malefyt *et al.*, 1991). This could have diluted the IL-10 production capacity measurement. Another explanation is that the relation might be confounded. Firstly, depletion of muscle tissue by malnutrition or disease could have disrupted the beneficial role of IL-10 production capacity in muscle repair and maintenance. As BMI is likely to reflect muscle mass in this lean population, the stronger relation between IL-10 gene variants and handgrip strength in the higher BMI stratum points at this possibility. Secondly, infectious diseases might have interfered with our measurements of IL-10 cytokine production capacity. Earlier we have shown that infectious diseases are highly endemic in the research area and induce a pro-inflammatory immune response (Boef *et al.*, 2013). The relation between the IL-10 gene variants and cytokine production capacity was less outspoken in the smaller group with available data on handgrip strength, possibly due to such environmental factors (May *et al.*, 2009a). Thirdly, physical activity attenuates the production capacity of monocytes (Walsh *et al.*, 2011), but meanwhile improves muscle strength (Ferreira *et al.*, 2012). In this population, physical activity is of vital importance due to the manual labour in farming and housekeeping that is necessary for subsistence up to the highest ages. Fourthly, while we measured cytokine production in whole blood, muscle tissue is recognised to be a cytokine producing organ itself. Although little has been reported about the muscle-specific production of IL-10, the IL-10 gene might exert its effects in muscle tissue in an autocrine and paracrine manner (Pedersen & Febbraio, 2012). Such a mechanism would not be revealed by the analysis of cytokine production capacity in whole blood samples.

While we observed no relation between IL-10 production capacity and handgrip strength, we observed a positive relation between TNF-α production capacity and handgrip strength in men, but not in women. This finding is in agreement with our research in Caucasians (Beenakker *et al.*, 2013). There we found a men-specific positive relation between TNF-α production capacity upon stimulation with LPS, a Toll-like receptor (TLR) 4 agonist, and muscle mass and strength. A women-specific positive relation was found between TNF-α production capacity upon stimulation with Pam3Cys-SK4, a

TLR-2/1 receptor agonist, and muscle mass. These findings indicate that in skeletal muscle tissue the TRL-4 pathway is predominant in men and the TLR-2/1 pathway is predominant in women.

Our study has some limitations. Firstly, as in all genetic association studies, we cannot exclude that the IL-10 gene variants are in linkage disequilibrium with variants of other genes that affect handgrip strength. However, we have previously described that this is unlikely, because resequencing of the IL10 gene region and its surroundings did not result in any variants additional to the SNPs that were genotyped in the IL-10 gene (Kuningas *et al.*, 2009). Secondly, we did not document possible epigenetic variation in the IL-10 gene. There is growing evidence that caloric intake and dietary composition modify epigenetic marks (Li, Daniel & Tollefsbol, 2011; McKay & Mathers, 2011), which can influence transcription of the IL-10 gene in immune cells (Villagra, Sotomayor & Seto, 2010). Malnutrition, being common in the research area (Ghana Health Service, 2005; Hesselberg & Yaro, 2006; Ghana Statistical Service, 2009; Koopman *et al.*, 2012), could thereby affect the relation between the IL-10 gene and muscle strength. Lastly, this is a cross-sectional study, while it would be valuable to associate IL-10 gene variants with longitudinal decline in handgrip strength over age.

In conclusion, this study shows that IL-10 gene variants associate with the production capacities of IL-10 and TNF-α and strongly relate to handgrip strength in rural Africa. A haplotype reflecting a pro-inflammatory immune response was associated with higher muscle strength, while a haplotype reflecting an anti-inflammatory immune response was associated with lower muscle strength, especially after exclusion of individuals with underweight. Future studies are needed to elucidate whether variants of the IL-10 gene determine handgrip strength through their role in the repair of skeletal muscle tissue directly or indirectly through their role in the defence against infectious diseases.

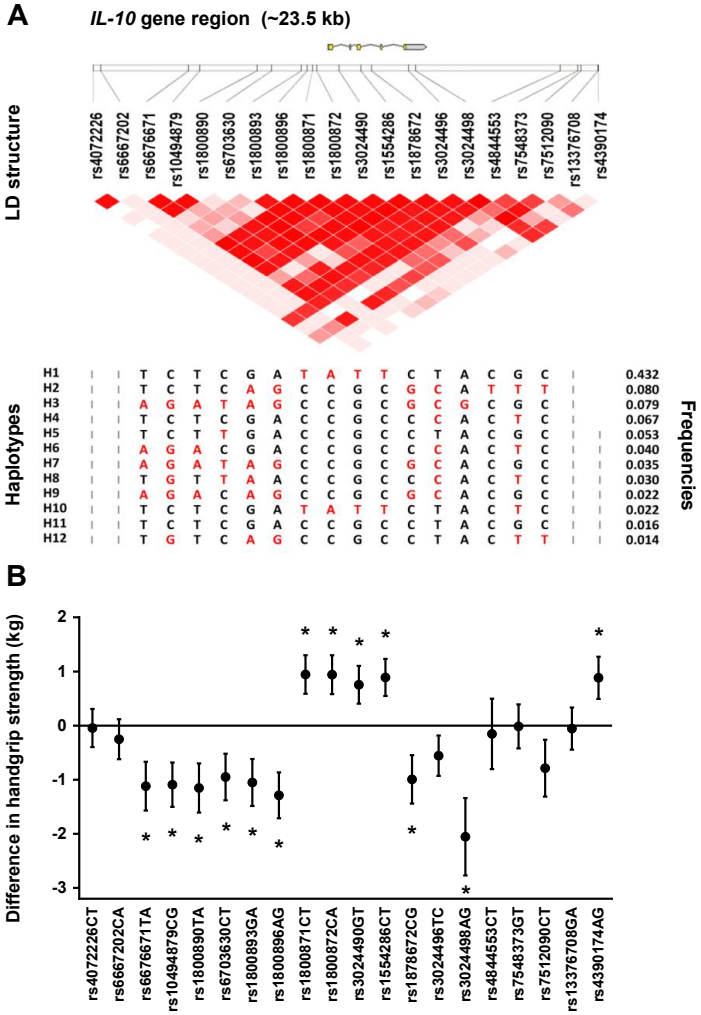

Figure 7.2. Association of IL-10 gene SNPs with handgrip strength. (A) A schematic overview of the IL-10 gene region with the locations of the genotyped single nucleotide polymorphisms (SNPs) indicated by vertical lines. Pair-wise linkage disequilibrium (LD) as observed in the entire genotyped population (n = 4336) is depicted in red. Population frequencies of the different haplotypes (if > 1%) are presented with the minor alleles of each SNP indicated in red (Kuningas *et al.*, 2009). (B) The relation between the minor allele of each IL-10 gene SNP and handgrip strength for individuals of whom IL-10 gene variants and handgrip were known (n = 554). Handgrip strength is expressed as the deviance from the population's mean in kilograms (kg) with standard error bars for carriers of at least one copy of the minor allele, adjusted for age, sex, tribe, household, and height (* $p < 0.05$).

Table 7.4. Locations and minor allele frequencies of the IL10 gene SNPs in the study population and in the entire genotyped population.

			Study population (*n* = 554)		Entire genotyped population (*n* = 4336)	
***IL-10* SNPs**	**Alleles[a]**	**Location**	**MAF**	**HWE**	**MAF**	**HWE**
rs4072226	C/T	promoter	0.463	0.936	0.456	0.865
rs6667202	C/A	promoter	0.499	0.851	0.484	0.602
rs6676671	T/A	promoter	0.195	0.870	0.200	0.832
rs10494879	C/G	promoter	0.269	0.823	0.284	0.025
rs1800890	T/A	promoter	0.192	0.822	0.201	0.865
rs6703630	C/T	promoter	0.206	0.579	0.220	0.066
rs1800893	G/A	promoter	0.256	0.005	0.284	0.196
rs1800896	A/G	promoter	0.260	0.064	0.284	0.401
rs1800871	C/T	promoter	0.509	0.984	0.470	0.302
rs1800872	C/A	promoter	0.506	0.811	0.472	0.034
rs3024490	G/T	intron	0.523	0.695	0.484	0.013
rs1554286	C/T	e/i boundary	0.500	0.433	0.468	0.157
rs1878672	C/G	intron	0.228	0.072	0.244	0.612
rs3024496	T/C	exon	0.393	0.928	0.425	0.043
rs3024498	A/G	exon	0.066	0.904	0.083	0.129
rs4844553	C/T	3' UTR	0.080	0.963	0.096	0.084
rs7548373	G/T	3' UTR	0.282	0.790	0.297	0.190
rs7512090	C/T	3' UTR	0.126	0.681	0.132	0.084
rs13376708	G/A	3' UTR	0.320	0.784	0.327	0.273
rs4390174	A/G	3' UTR	0.290	0.981	0.282	0.630

[a] Major/minor allele. e/i boundary: exon/intron boundary. MAF: minor allele frequency. HWE: *p*-values for Hardy-Weinberg equilibrium.

Table 7.5. Minor allele frequencies of IL-10 gene SNPs for the included tribes

	Minor allele frequencies						
***IL-10* SNPs**	**Bimoba (n=394)**	**Kusasi (*n* = 128)**	**Mamprusi (*n* = 14)**	**Fulani (*n* = 3)**	**Busanga (*n* = 10)**	**Other (*n* = 3)**	***p***
rs4072226	0.443	0.516	0.536	0.500	0.400	0.500	0.29
rs6667202	0.524	0.429	0.464	0.250	0.550	0.400	0.10
rs6676671	0.186	0.213	0.250	0.000	0.300	0.200	0.25
rs10494879	0.367	0.276	0.286	0.167	0.350	0.300	0.48
rs1800890	0.183	0.211	0.250	0.000	0.300	0.125	0.33
rs6703630	0.194	0.238	0.250	0.000	0.300	0.300	0.12
rs1800893	0.261	0.262	0.154	0.167	0.222	0.000	0.20
rs1800896	0.260	0.264	0.231	0.177	0.300	0.200	0.87
rs1800871	0.509	0.508	0.429	0.833	0.550	0.500	0.79
rs1800872	0.506	0.504	0.429	0.833	0.550	0.500	0.77
rs3024490	0.527	0.512	0.429	0.833	0.556	0.500	0.92
rs1554286	0.503	0.500	0.364	0.833	0.500	0.333	0.71
rs1878672	0.227	0.238	0.154	0.167	0.462	0.100	0.80
rs3024496	0.398	0.399	0.423	0.177	0.350	0.250	0.34
rs3024498	0.059	0.089	0.071	0.000	0.001	0.000	0.56
rs4844553	0.087	0.075	0.036	0.000	0.000	0.000	0.044
rs7548373	0.300	0.258	0.250	0.000	0.056	0.200	0.009
rs7512090	0.141	0.105	0.105	0.038	0.000	0.000	0.003
rs13376708	0.308	0.352	0.357	0.167	0.250	0.600	0.23
rs4390174	0.275	0.321	0.321	0.667	0.350	0.200	0.19

Differences in minor allele frequencies between tribes have been tested by the linear-by-linear association test.

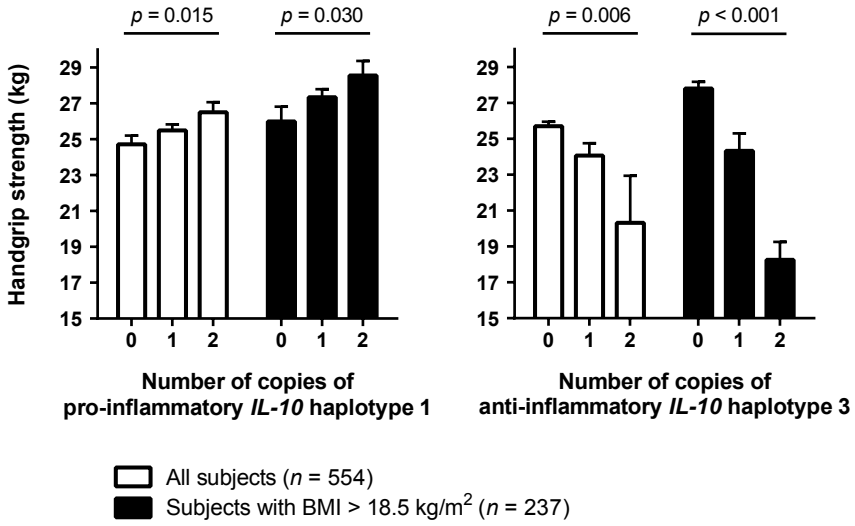

Figure 7.3. Association of IL-10 gene haplotypes with handgrip strength in all subjects and in subjects with a normal BMI. Handgrip strength for individuals of whom IL-10 gene variants and handgrip were known (n = 554) presented as means with standard error bars, adjusted for age, sex, tribe, household, and height (p values for trend). A BMI of 18.5 kg/m^2 or lower is regarded as underweight (Shetty & James, 1994; World Health Organization, 2003). For the haplotype structures and frequencies, see Figure 7.2A.

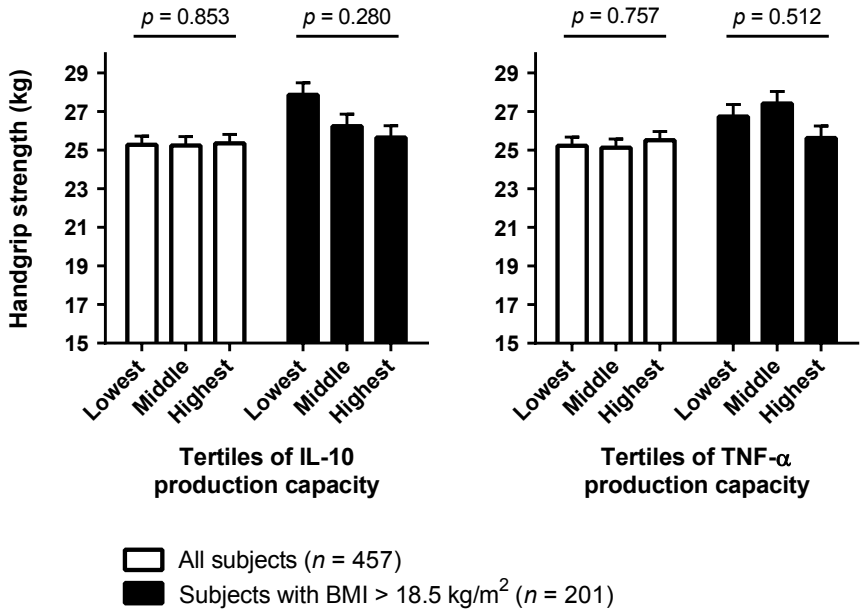

Figure 7.4. Association of cytokine production capacities with handgrip strength in all subjects and in subjects with a normal BMI. Handgrip strength for individuals of whom IL-10 gene variants, cytokine production capacities, and handgrip strength were known (n = 457) presented as means with standard error bars, adjusted for age, sex, tribe, household, and height (p values for trend). A BMI of 18.5 kg/m^2 or lower is regarded as underweight (Shetty & James, 1994; World Health Organization, 2003).

Chapter 8

Discussion

Perspectives

The aim of this thesis is to investigate the role of chronic inflammation as well as acute inflammatory response on muscle aging.

Overview of the results

We began our study by examining the role of chronic inflammation on muscle aging. This was investigated in patients with rheumatoid arthritis (RA), a chronic disease characterized by high levels of circulating inflammatory mediators. We hypothesized that if chronic inflammation has an important role in muscle aging, that we then would find signs of accelerated muscle aging in RA patients. Indeed, we found that the presence and duration of a chronic inflammatory state like rheumatoid arthritis is strongly associated with low muscle strength. However, in muscle tissue of RA patients we did not find histological signs of accelerated muscle aging. Neither did we find more type II specific muscle fiber atrophy, nor more accumulation of disposal granules like lipofuscin, nor a lower amount of muscle stem cells (satellite cells) per muscle fiber. One interpretation of these data is that loss of muscle strength and inflammation does not lead to accelerated aging of muscle cells and that chronic inflammation leads to loss of muscle strength via other mechanisms such a pain and low vascularisation of muscle. However, these negative findings motivated us to search for a more complex relation between inflammation and muscle aging as the initiation, regulation, and effect of inflammation is a complex nature in itself.

We asked ourselves whether chronic inflammation and acute inflammatory response are two mutually dependent or independent endotypes. We found that circulating markers of inflammation (as an estimate of chronic inflammation) and cytokine production response (as an estimate of acute inflammatory response) are not correlated. Both are independently and positively associated with mortality due to cardiovascular disease (which we took as example disease). Having confirmed that the acute inflammatory response is an independent endotype, we addressed the potential confounding effect of sex differences in the relation between acute inflammatory response and occurrence of disease. We found that men have a substantially higher cytokine production response compared to women and that this is mainly explained by sex differences in monocyte concentrations — a fact that is frequently overlooked in published research.

Finally, while taking sex differences into account, we investigated the relation between cytokine production response and muscle mass and strength.

In Dutch elderly we found that a higher pro-inflammatory cytokine production response was positively associated with higher muscle mass and strength. We further explored this association by investigating the relation between *interleukin-10* (IL-10) gene variants, known to be associated with cytokine production response, and muscle strength. In African elderly we found that a haplotype reflecting a high *pro*-inflammatory cytokine production response was associated with *higher* muscle strength, while a haplotype reflecting a high *anti*-inflammatory cytokine production response was associated with *lower* muscle strength. The finding in this genetic study contributes to an interpretation of the relation between a pro-inflammatory cytokine production response and muscle strength which is free from confounders and reverse causality.

We conclude that high chronic inflammation is associated with low muscle strength, while high acute pro-inflammatory response is associated with high muscle strength.

Reflection

This conclusion, that chronic inflammation and acute inflammatory response are not only two independent endotypes, but that they have a completely opposite relationship to muscle strength, appears counter-intuitive. We may explain this by the following observations.

Firstly, the two types of inflammation differ in duration. We used a whole blood stimulation assay to measure cytokine production response as an estimate of acute inflammatory response. This assay measures the amount of cytokines that are produced within 24 hours after a stimulus. In comparison, chronic inflammation during the aging process develops over years to decades (Franceschi & Campisi, 2014). Chronic inflammation in RA has a duration that is at least as long as the formal disease duration, which in the RA patients described in chapter 4 was on average 22.6 years (standard deviation 13.0). The large difference in duration between the two types of inflammation possibly reflects the difference between controlled inflammation and uncontrolled inflammation. During controlled inflammation the tissue returns to homeostasis, while in uncontrolled inflammation this does not happen and therefore inflammation persists. Mice studies revealed that the resolution of acute inflammation is an active process involving specific molecules called resolvins, protectins, and lipoxins (Serhan, Chiang & Van Dyke, 2008). This resolution process is considered to be a distinct process from the anti-inflammatory process, because these pro-resolution molecules also promote the uptake and clearance of apoptotic cells and microorgan-

isms by macrophages in inflamed sites. It might be that chronic inflammation arises from defects in the signalling pathway or synthesis of the pro-resolution molecules. However, this has not yet been proven, and pro-resolution molecules have also not yet been related to muscle mass and strength.

Secondly, the two types of inflammation differ in source. Cytokines produced during the acute inflammatory response have as their main source monocytes (Damsgaard *et al.*, 2009a). Cytokines involved in chronic inflammation have as their main source a wide variety of cell types, including lymphoid cells as well as non-lymphoid cells such as endothelial cells, fibroblasts, and adipocytes (Naka, Nishimoto & Kishimoto, 2002). Activated monocytes migrate into the muscle tissue, where they stimulate muscle stem cells (satellite cells) and muscle regeneration (Arnold *et al.*, 2007). Non-lymphoid cells have a completely different relation with muscle tissue. Atherosclerosis due to endothelial dysfunction, muscle fibrosis formed by fibroblasts, and obesity formed by adipocytes all have a negative association with muscle mass and strength (Moyer & Wagner, 2011; Budui, Rossi & Zamboni, 2015).

Finally, the two types of inflammation differ in their actual versus potential inflammation. Acute inflammatory response measurements reflects the in vitro capacity of immune cells to produce cytokines, not the actual in vivo production of cytokines, which is reflected in measurements of chronic inflammation. Acute inflammatory response is highly innate determined (De Craen *et al.*, 2005), while chronic inflammation is acquired over decades. How chronic inflammation is acquired remains largely unknown, but it is commonly thought to be the result of accumulation of cellular damage and has been associated with a wide variety of age-related diseases (Franceschi & Campisi, 2014). It is still unclear whether chronic inflammation plays a causal role in muscle aging or is only an epiphenomenon. The role of acute inflammatory response in muscle aging could be direct, but also indirect through its effect on a wide variety of age-related diseases. These diseases include sepsis (Wilhelm *et al.*, 2002), type 2 diabetes, metabolic syndrome (Van Exel *et al.*, 2002), osteoarthritis (Riyazi *et al.*, 2005), rheumatoid arthritis (De Vries-Bouwstra *et al.*, 2007), multiple sclerosis (De Jong *et al.*, 2000, 2002), lupus erythematosus (Van der Linden *et al.*, 2000), depression (Van den Biggelaar *et al.*, 2007), and Alzheimer's disease (Van Exel *et al.*, 2009).

Clinical implications

The observations described in this thesis highlight differences between chronic inflammation and acute immune response in relation to muscle mass and strength.

In clinical practice, the estimation of chronic inflammation by measurements of high-sensitivity CRP has been extensively studied in relation to cardiovascular risk management and appeared to be of additive value (Kaptoge *et al.*, 2012). Large prospective studies are needed to investigate its additive value for "sarcopenia" risk management.

To our knowledge, cytokine production response has until now only been measured for research purposes and not for clinical practice. The currently used stimulation assays suffer from large batch-effects, preventing comparisons between samples from different batches and from different laboratories. Moreover, the stimulation assays used in our research are very time consuming. A more simplified and more standardized stimulation assay has to be developed before the measurement of cytokine production response could be used in the clinical practice for the prediction of low muscle strength.

Future directions

Further studies are needed to investigate the role of acute immune response in maintaining muscle strength. Until now, the majority of studies investigating the relation between the innate immune system and the muscle have been performed in mice and not in humans. The innate immune systems of mice and humans have essential differences. In particular, gene expression profiles of classically and alternatively activated monocytes differ between mice and humans (Ziegler-Heitbrock, 2014). Therefore, the observation in mice that upon muscle injury macrophages first secrete pro-inflammatory cytokines to stimulate satellite cell growth and then secrete anti-inflammatory cytokines to stimulate satellite cell proliferation needs to be confirmed in humans (Arnold *et al.*, 2007). More research into the interaction between innate immune cells and satellite cells in humans would provide more insight into the mechanisms underlying the association we found between cytokine production response and muscle strength.

In our research, we measured cytokine production response upon stimulation with LPS in whole blood. However, it has not yet been investigated how well cytokine production response in whole blood corresponds to cytokine production response in the muscle upon muscle injury. Furthermore, we measured cytokine production response using a ligand secreted by bacteria. Although it is known that endogenous danger signals like high mobility group box 1 (HMGB1) elicit the same inflammatory responses as pathogens (Bianchi & Manfredi, 2009), studies are needed to make clear which dose of pathogens used during a whole blood stimulation assay corresponds to which dose of endogenous danger signals excreted during muscle injury. Further

studies are also needed to investigate the relation between acute inflammatory response of monocytes in the whole blood in relation to anti-inflammatory activated and pro-inflammatory activated macrophages in the muscle.

In order to further explore a potential causal relation between acute inflammatory response in muscle mass and strength more experimental research is needed. Although cytokine production response, as a measure of acute inflammatory response, is highly genetically determined (De Craen *et al.*, 2005), small clinical trials suggest that it is possible to modify cytokine production response. For instance, it has been shown that cytokine production response can be decreased by the intake of fish oil (Damsgaard *et al.*, 2009b) and red wine (Johansen *et al.*, 1999), but can also be increased by anti-TNF therapy (Kayakabe *et al.*, 2012), IL-1RA therapy (Smith *et al.*, 2012), and nonsteroidal anti-inflammatory drugs (Page *et al.*, 2010). Furthermore, the cytokine production of monocytes and/or macrophages are known to be regulated by the central nerve system through the alpha-7 nicotinic acetylcholine receptor pathway (Wang *et al.*, 2003). Through this pathway acetylcholine released by electrical stimulation of the vagal nerve, nicotine, and the selective alpha-7 nicotinic acetylcholine receptor agonist GTS-2 all suppres the amount of produced cytokines by monocytes and/or macrophages (Borovikova *et al.*, 2000; Rosas-Ballina *et al.*, 2009). Future research could make use of these interventions to investigate the effect of changes in the acute inflammatory response on muscle strength and mass.

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Summary in Dutch

Doel van het proefschrift

Het doel van dit proefschrift is de rol van chronische ontsteking en van de acute ontstekingsreactie in spierveroudering te onderzoeken.

Chronische ontsteking en spierveroudering

We onderzochten patiënten met rheumatoide arthritis (RA), een chronische ziekte die gekarakteriseerd wordt door hoge ontstekingswaardes die in het bloed circuleren. Onze hypothese was dat, als chronische ontsteking een belangrijke rol heeft in spierveroudering, dat we dan tekenen van versnelde spierveroudering in RA-patiënten zouden vinden. Inderdaad vonden we dat, ten opzichte van de algemene bevolking, de aanwezigheid en duur van de ontstekingsziekte RA sterk geassocieerd is met lage spierkracht. Dit werd gevonden in gepoolde data uit 185 studies betreffende 10149 proefpersonen (*hoofdstuk 2*). Echter, in het spierweefsel van RA-patiënten vonden wij, ten opzichte van spierweefsel van controlepatiënten met artrose (OA), geen histologische kenmerken van versnelde spierveroudering. We vonden noch meer type-II spiervezelatrofie, noch meer accumulatie van afvalgranulen zoals lipofuscine, en ook geen lagere hoeveelheid spierstamcellen (satellietcellen) per spiervezel. Deze bevinding werd gedaan gebruikmakend van spierbiopsie data van 10 RA en 27 OA-patiënten die een electieve knieoperatie ondergingen (*hoofdstuk 3*). Een interpretatie van deze data is dat verlies van spierkracht en ontsteking niet leiden tot versnelde spierveroudering. Chronische ontsteking leidt tot spierverlies via andere mechanismen zoals pijn en verminderde vasculatie van de spier. Deze negatieve resultaten motiveerden ons om onderzoek te doen naar een meer complexe relatie tussen ontsteking en spierveroudering omdat het begin van onsteking, de regulatie en het effect van ontsteking een complex geheel vormen.

Chronische ontsteking en de acute ontstekingsreactie

We vroegen onszelf af of chronische ontsteking en de acute ontstekingsreactie twee onderling afhankelijke of ónafhankelijke endotypes zijn. We vonden dat circulerende ontstekingsstoffen (als schatter voor chronische ontsteking) en *cytokine production response* (als schatter voor de acute ontstekingsreactie) niet met elkaar correleren. Beiden bleken onafhankelijk van elkaar positief geassocieerd te zijn met sterfte door hart en vaatziekten (een ziekte die we als voorbeeldziekte namen). Dit werd gevonden gebruikmakend van gegevens van 403 proefpersonen van de “PROspective study of Pravastatin in Elderly at Risk” (PROSPER) (*hoofdstuk 4*). Nadat we dus hadden aange-

toond dat de acute ontstekingsreactie een onafhankelijk endotype is, onderzochten we de potentiële vertekening door geslachtsverschillen in de relatie tussen de acute ontstekingsreactie en ziektes. We vonden dat mannen ten opzichte van vrouwen een substantieel hogere *cytokine production response* hebben. Dit verschil werd grotendeels verklaard door geslachtsverschillen in monocytconcentraties in het bloed, een feit waar in onderzoek vaak overheen gekeken wordt. Voor dit onderzoek maakten we gebruik van data van 4020 deelnemers van 15 studiepopulaties van de algemene bevolking en van populaties van patiënten met specifieke ziektes (*hoofdstuk 5*).

Acute ontstekingsreactie en spierveroudering

Rekening houdend met geslachtsverschillen, onderzochten we tenslotte de relatie tussen enerzijds de *cytokine production response* en anderzijds spiermassa en spierkracht. Uit muizenstudies was inmiddels al gebleken dat de acute ontstekingsreactie van monocyten van cruciaal belang is voor het stimuleren van spierstamcellen en het herstel van beschadigd spierweefsel. In Nederlandse ouderen vonden we dat een hogere *pro-inflammatory cytokine production response* geassocieerd is met een hogere spiermassa en spierkracht. Dit werd gevonden gebruikmakend van data van de "Leiden LangLeven" studie betreffenden 191 mannen en 195 vrouwen die afstammelingen zijn van langlevende familieleden of partners van afstammelingen (*hoofdstuk 6*). Vervolgens onderzochten wij deze relatie op een dieper niveau. Dit deden wij door onderzoek te doen naar de relatie tussen *interleukine-10* (IL-10) genvariaties (geassocieerd met *cytokine production response*) en spierkracht. In Afrikaanse ouderen vonden we dat een genvariatie voor een hogere *pro-inflammatory cytokine production response* geassocieerd is met hogere spierkracht en een genvariatie voor een hogere *anti-inflammatory cytokine production response* geassocieerd is met lagere spierkracht. Dit onderzoek werd gedaan in 554 ouderen uit platteland in Ghana waar spierkracht van vitaal belang is en waar de mensen verrijkt zijn met genvariaties voor een hoge *pro-inflammatory cytokine production response* (*hoofdstuk 7*). De bevindingen van deze genetische studie dragen bij aan een interpretatie van de relatie tussen *pro-inflammatory cytokine production response* en spierkracht die vrij is van vertekening en van omgekeerde causaliteit.

Conclusie

Wij concluderen dat de chronische ontsteking geassocieerd is met lage spierkracht, terwijl de acute ontstekingsreactie geassocieerd is met hoge spierkracht.

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List of Publications

1. K. G. M. Beenakker, C. H. Ling, C. G. M. Meskers, A. J. M. de Craen, Th. Stijnen, R. G. J. Westendorp, and A. B. Maier, *Patterns of muscle strength loss with age in the general population and patients with a chronic inflammatory state*, Ageing Research Reviews **9**, 431–436 (2010) [chapter 2].

2. B. J. Duijnisveld, A. Bigot, K. G. M. Beenakker, D. M. Portilho, V. Raz, H. J. L. van der Heide, C. P. J. Visser, S. Chaouch, K. Mamchaoui, R. G. J. Westendorp, V. Mouly, G. S. Butler-Browne, R. G. H. H. Nelissen, and A. B. Maier, *Regenerative potential of human muscle stem cells in chronic inflammation*, Arthritis Research & Therapy **13** R207 (2011).

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9. K. G. M. Beenakker, S. Chen, M. P. Rozing, members of the PROSPER-group, R. G. J. Westendorp, and A. B. Maier, *Immune responsiveness associates with cardiovascular mortality independent of circulating markers of inflammation* (in preparation) [chapter 4].

* equal contribution

Curriculum Vitæ

Karel Beenakker was born in Eindhoven, The Netherlands, on 21 October 1986. After his family moved to Voorschoten, he attended secondary school at the Stedelijk Gymnasium in Leiden. During the 5th and 6th grade he performed his school research project (*profielwerkstuk*) on "The causes of aging". After graduation in 2005 he started medical school at Leiden University Medical Center (LUMC). In 2006 he won the Einthoven Student Award for a research project on electrocardiograms. In 2006 he was selected for the MD/PhD track, a program for medical students who start with their PhD research already during medical school. He started this honours track at the LUMC department of Geriatrics and Gerontology. In 2011 he graduated from medical school (*cum laude*) and received an unrestricted grant from the Board of the LUMC for a two year appointment as a PhD student, resulting in the research reported in this thesis. In December 2013 he started his specialist training in Psychiatry at the LUMC and Rivierduinen.
Karel lives in Leiden with his wife Anne-Marijn. They have two children, Max[†] and Jacinta.