# The role of inflammation in muscle aging

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# Chapter 3

# Muscle characteristics in patients with an underlying inflammatory state

Published as: K.G.M. Beenakker, B.J. Duijnisveld, H.M.J. van der Linden, C.P.J. Visser, R.G.J. Westendorp, G. Butler-Brown, R.G.H.H. Nelissen, A.B. Maier, *Muscle & Nerve* **46** (2012) 204–209.

## 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 $\mu$m and 5 $\mu$m 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 $\mu$m 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 $\mu$m 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 $\mu$m 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

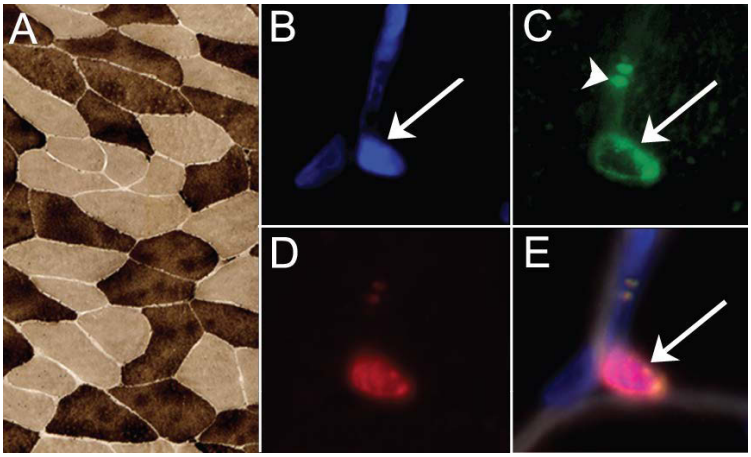


**Figure 3.1.** Images of muscle cross-sections stained for muscle fiber typing and satellite cell identification by multi-labeling. A: Staining of type I (white) and type II (black) muscle fibers B: DAPI staining (blue), C: N-CAM staining (green), D: Pax7 staining (red), E: Combination of DAPI, N-CAM,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

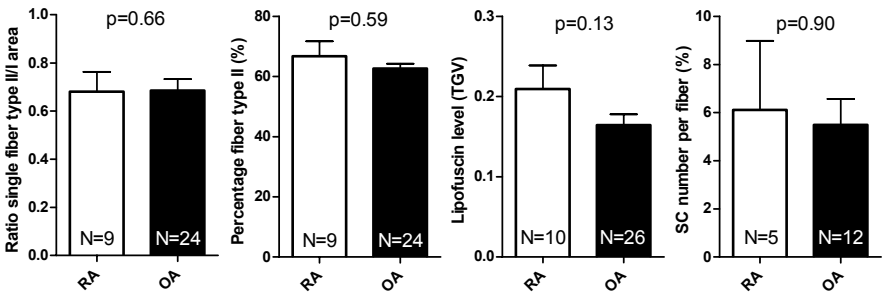


**Figure 3.2.** Ratio of type II and type I single fiber areas, percentage type II fibers, lipofuscin level and satellite cell number per fiber in muscle tissue from RA and OA patients. Results are given as mean and standard error. P-values are calculated using linear regression analysis adjusted for 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-$\alpha$ 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.