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Myosteatorsis and Myofibrosis: relationship with aging, inflammation and insulin resistance

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Abstract

The mechanisms impairing muscle quality and leading to myofibrosis (MF) and myosteatorsis (MS), are incompletely known.

In biopsies of paraspinous muscle (PM) of 16 healthy elderly men undergoing elective vertebral surgery, we histologically determined the area of MF and MS expressed as muscle quality index (MQI), in order to investigate the relation between them, as well as the main predictors of muscle quality. Total PM muscle area and intermuscular adipose tissue (IMAT) were evaluated by MRI and body composition by DXA. Circulating fasting glucose, insulin, hs-CRP, leptin, adiponectin and IL-6 were measured and HOMA index calculated. Quantification of gene expression in PM and in subcutaneous adipose tissue (SAT) overlying the muscle was performed by real time-PCR. The degree of MS and MF were significantly and positively related to each other and positively associated with BMI, waist circumference, FM and FM% as well as with the amount of IMAT. Leptin levels were positively associated with MF and MS, whereas hs-CRP was significantly related only to MF. In backward regression analyses, larger waist circumference and smaller PM muscle area explained 90% of MF variance, whereas leptin predicted about 80% of MS variance. IL-6 expression in SAT was significantly higher in participants with higher MQI values. In PM biopsies we found significantly higher expression of SOCS-3 and a trend toward higher expression of myostatin with greater degrees of MQI.

MS and MF are related phenomena that concur to alter muscle quality with aging and both should be considered in the evolution of sarcopenia.

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CONFLICT OF INTEREST STATEMENT

All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence this scientific work.

Keywords

Sarcopenia; muscle quality; fat infiltration; fibrosis; aging

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INTRODUCTION

Health consequences of sarcopenia have been the object of extensive study in the past two decades (Janssen 2010, Glass 2010). Sarcopenia is the age-associated loss of skeletal muscle mass and function, and is characterized not only by quantitative changes in skeletal mass, but also by qualitative muscle changes that contribute an increased risk of functional impairment, physical disability and frailty (Janssen 2010, Glass 2010).

The role of increased muscle fat infiltration, also called myosteatosis (MS), has been object of intense investigation, especially as it relates to sarcopenia (Goodpaster 2001, Gallagher 2005, Petersen 2003, Visser 2005, Beasley 2009, Miljkovic 2010). MS increases with age and adiposity (Goodpaster 2001, Gallagher 2005) and is strongly related to metabolic abnormalities (Petersen 2003), poorer strength and scores on performance tests (Visser 2005), as well as incident mobility disability (Visser 2005). In the Health ABC Study population, the degree of thigh intermuscular fat was significantly associated with higher levels of inflammatory markers (Beasley 2009).

Aging of skeletal muscle is also associated with MF, an increase in fibrous connective tissue (Goldspink 1994), and an impairment of muscle regenerative potential (Brack 2007), manifested by a replacement of muscle by fibrous connective tissue. Sections from muscles of mice at different ages showed accumulation of collagen with age, similar to those of mdx dystrophic mice, although to a lesser extent (Goldspink 1994). Muscle stem cells from aged mice tend to convert more to a fibrogenic lineage compared to cells from young muscles (Brack 2007). The pathways involved in this impaired muscle regeneration and enhanced fibrotic response are still incompletely known (Brack 2007, Graham 2010, Trenz 2010, Zhu 2007).

Few papers using mouse models previously described a clear association between muscle fibrosis and aging (Goldspink 1994, Brack 2007, Trenz 2010), but a lack of studies in human must be observed. Functional consequences of myofibrosis (MF) could be relevant as an increase in MF can lead to a decrease in muscle strength and elasticity as well as to reduced blood supply of muscle fibers, further increasing muscle fiber atrophy (Huard 2002, Jarvinen 2002). Furthermore, both MS and MF are associated with poorer muscle quality. However, little is known of the relationship between MS and MF. The aim of this paper was to investigate the relation between MS and MF, as well as the main predictors of histologically determined muscle quality during aging. To test this hypothesis we selected a sample of healthy elderly men undergoing elective vertebral surgery. In all these participants we obtained specimens of paraspinal muscle (PM) and subcutaneous adipose tissue (SAT) overlying the muscle, and related histologically evaluated MS and MF to MRI-determined characteristics of PM including intermuscular adipose tissue (IMAT), as well as systemic and local inflammatory biomarkers.

METHODS

A total of 16 men, aged between 58 and 80 years (70.3 ± 7.4 years, mean $\pm$ SD) with BMI ranging from 24.5 to 44 Kg/m^2 (30.4 ± 4.8 Kg/m^2 , mean $\pm$ SD) were studied (table 1).

All participants were selected among those undergoing elective surgery for medullar lumbar stenosis in the Department of Neurosurgery of our Hospital.

All participants had been weight-stable over the previous 6 months, in general good health, and had no history of type 2 diabetes. Physical activity level was recorded with a scale of 1 to 3 (3 being regular physical activity level, 2 being occasional, 1 being low physical activity level). The average activity level in our study population was 1.3 (ranging from 1 to 2). None showed ADL deficit. Participants receiving steroid and/or immunosuppressive medications in the previous 6 months and using anti-inflammatory medications more than once a week were excluded.

All participants gave their informed consent and the experimental protocol was approved by the Ethical Committee of our Hospital.

Anthropometry and Body composition measurements

BMI was computed as weight divided by stature squared (Kg/m^2); waist circumference was obtained as previously described (Zoico et al., 2010).

Body composition was studied using Dual energy X-ray absorptiometry (DXA) (Hologic QDR 4500, Waltham, USA) fan beam with software version 8.21. Total body fat (FM) was expressed in Kg and as percentage of body weight (FM %). Lean total body mass (LFM) was the difference between fat-free mass (FFM) and bone mineral mass and expressed in Kg. The CV for double determinations in 10 elderly subjects was 1% for FM and 1.3% for LFM.

Study of muscle fat infiltration with MRI

Participants were positioned supine on a MR scanner and transverse sections were obtained at L3-L4 disk level using a 1.0-T Siemens Armony Expert (Siemens AG, Erlangen, Germany). A high resolution T1-weighted sequence was obtained for every participant. PM measurements on the right and left side (mean values) were analyzed (Käser et al., 2001), using Sliceomatic image software (version 4.2; Tomovision, Montreal, Canada).

Adipose tissue deposition in skeletal muscle, defined as IMAT, was considered as adipose tissue area visible between muscle groups and beneath the muscle fascia (Song et al., 2004). Intraclass correlation coefficients for IMAT was higher than 0.97 (Rossi et al., 2010).

Subcutaneous Fat and Muscle biopsies

SAT and PM biopsies were taken at the beginning of vertebral surgery in the site of surgical incision (L3–L4), in a standardized manner. Fat and muscle tissues were immediately frozen for gene expression analysis with real time-PCR.

Total RNA was extracted from SAT and PM biopsies with Qiazol (Qiagen) and chloroform and the aqueous phase purified using an RNeasy Mini or Micro Kit (Qiagen). 15 ng of total RNA for each gene were reverse transcribed into cDNA in 20 µl reactions with the IScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Aliquots of the reverse transcriptase were PCR-amplified with QuantiTect SYBR Green PCR kit (Qiagen) and with Quantitect primer Assays (Qiagen) for each gene in the iCycler thermocycler (Bio-Rad, Hercules, CA). Gene expression in both tissues was normalized against 18S rRNA for each sample and performed in triplicate (software Bio-Rad iQ5).

Histological and morphometric analysis of MF and MS in muscle biopsies

PM biopsies were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 overnight at 4°C, dehydrated, cleared and paraffin embedded. 3µm thick sections from three different levels (every 200µm) were cut to obtain sections representative of the whole biopsy. All sections were stained with Hematoxylin and Eosin (H&E) for histological and morphometric analysis. The morphological observations were performed with a Nikon Eclipse 80i light microscopy (Nikon, Japan).

For each patient three level sections of stained PM were used for the measurement of area occupied by collagen fibers (MF area) and by fat infiltration (MS area) (Fig 1). MF and MS area were measured using image analysis software (Lucia Image v.4.82, Nikon Instruments, Cz).

Results are given as mean percentage of MF area and mean percentage of MS area of the three levels of each muscle biopsy. We also calculated a Muscle Quality Index (MQI) by adding the mean percentage of MF area and the mean percentage of MS area and dividing by 2. This captures the degree of skeletal muscle substitution: the higher the index, the greater the amount of skeletal muscle area substituted by connective and adipose tissue.

Biochemical analyses

Venous blood samples were obtained after overnight fasting. Plasma glucose was measured using a glucose analyzer and plasma immune-reactive insulin by double antibody radioimmunoassay using a commercial kit. Insulin resistance was estimated by the homeostasis model assessment (HOMA) method. Circulating levels of leptin, adiponectin, and IL-6 were measured using specific ELISA kits. High sensitive-C reactive protein (hs-CRP) was measured with the immunoturbidimetric method.

Statistical analysis

Results are shown as mean values $\pm$ standard deviations. Test for nonparametric variables were used for variables that were not normally distributed. Pearson and Spearman correlations were used to test association between variables, as well as partial correlation. We restricted the main analyses of the study to the group of men with complete data and complete set of specimens collected during surgery (n=11). We performed backward regression analyses where MF or MS were the dependent variables and age, waist circumference, PM muscle area, leptin, hs-CRP, and HOMA index the independent variables. Finally participants were divided into two groups based on the degree of MQI; as

a cut-off, the 50th percentile of MQI was chosen (15%). Comparison of gene expression, stratified for MQI values was performed by analysis of variance (ANOVA). The level for statistical significance was $p < 0.05$. All statistical analyses were performed using the SPSS 16.0 for Windows.

RESULTS

We first examined the reciprocal relation between histological muscular changes, in particular between MS and MF, in the whole study sample ($n=16$). The degree of MS and MF were significantly related each other ($r=0.667$; $p=0.005$). No significant relation was found between age and MF or MS in the study sample, which may reflect the small sample size and to the narrow age range of participants.

Tables 2 and 3 include analyses conducted only in a group of men ($n=11$) with complete data for the main study variables.

In table 2 are presented simple correlations between MF and MS and different anthropometric and body composition variables, intermuscular adipose tissue (IMAT), adipokines, and indices of inflammation and insulin-resistance. MF was significantly and positively associated with BMI, waist circumference, FM and FM% as well as with the amount of IMAT (table 2). Similarly, as expected, MS was significantly and positively related with BMI and the amount of total and central body adiposity, as well as with IMAT (table 2). No significant relation was found between MF and MS and the amount of total and appendicular LM evaluated by DXA (table 2). However the area of PM measured by MRI was negatively correlated with MF, but not with MS, even after adjustment for body weight ($r = -0.820$; $p=0.002$).

In this sample of men, circulating leptin levels were positively associated with the degree of MF and MS, whereas there was only a trend for a negative association between adiponectin and MF and MS (table 2). IL-6 circulating levels did not show any association with MS and MF in this study; however hs-CRP was significantly related with MF (table 2). MS, but not MF, presented a positive correlation of borderline significance with insulin resistance, evaluated by HOMA (table 2).

We finally studied the main correlates of muscle quality, performing backward regression analyses where MF or MS were the dependent variables and age, waist circumference, PM muscle area, leptin, hs-CRP, and HOMA index the independent variables (table 3). The main correlates of MF were larger waist circumference and smaller PM muscle area, together determining almost 90% of variance ($R^2 0.892$) (table 3). Circulating leptin levels predicted about 80% of variance of MS, independently of age, total and central adiposity, inflammation, insulin resistance and muscle area (table 3).

We created an index of muscle quality, expressing the degree of skeletal muscle substitution by MS and MF, the Muscle Quality Index (MQI). We studied the association between gene expression in SAT and PM biopsies and the degree of MF and MS, evaluated by MQI (Fig 2). The expression of IL-6 in SAT was significantly higher in the group of elderly participants with higher values of MQI compared to those with lower values of the index

(Fig 2) whereas only a trend was found for a lower expression of adiponectin and VEGF in men with lower MQI values (Fig 2).

We finally evaluated the relation between gene expression in skeletal muscle biopsies and the degree of MF and MS, expressed as MQI (Fig 3). We found significant higher expression of mRNA of SOCS-3 in participants with higher MQI values, compared to those with lower values, and a trend toward higher expression of myostatin in participants with higher degrees of muscle substitution (Fig 3). Gene expression of IGF-1, as well as of MYOD e MURF-1, were lower, although not significant, in men with higher MQI values (Fig 3).

DISCUSSION

This study describes a strong relation between MS, a qualitative changes of skeletal muscle usually associated with aging and MS. In this sample of elderly participants, MS is associated with a significant increase in MF, which is inversely related to muscle area, even after adjustment for other variables of body composition. In our study not only the degree of MS but also the degree of MF is associated with a pattern of increased adiposity, with central fat distribution and a worse metabolic profile. Moreover higher values of MS are associated with higher levels of circulating leptin and insulin resistance, whereas higher values of MF seems to be related with higher levels of hs-CRP and leptin.

The study of the main predictors of muscle quality has been acknowledged as an emerging field in geriatric research. Several epidemiological studies have shown, with aging, there is a mismatch between the age-related decrease in muscle mass and in muscle power, suggesting the presence of progressive qualitative changes in muscle with age (Goodpaster et al., 2001, 2006; Visser et al., 2005).

Aging of skeletal muscle is associated not only with muscle atrophy and replacement of muscle by adipose tissue (Goodpaster et al., 2001, Gallagher et al., 2005), but also with an increase in fibrous connective tissue (Goldspink et al., 1994, Brack et al., 2007, Graham et al., 2010, Trenszt et al., 2010). In vitro satellite cells derived from muscles of aged mice showed an impairment of their muscle regenerative potential converting more frequently toward fibrogenic cells (Brack et al., 2007). This also occurred in vitro when young murine cells were exposed to serum from aged animals, suggesting also the importance of the systemic environment in determining the fate of muscle stem cells (Brack et al., 2007). To our knowledge only a few studies performed in animal rodents have been published on muscle aging and fibrosis but no data area available in humans (Goldspink et al., 1994; Brack et al., 2007; Graham et al., 2010; Trenszt et al., 2010; Zhu et al., 2007; Huard et al., 2002; Jarvinen et al., 2002). Recently microarray data showed that Duchenne Muscular Distrophy and aging may share common gene responses and pathways, in particular factors that may coordinate either the immune/fibrosis responses or the mitochondrial metabolism (Baron 2011).

In the present study our data shows the existence of a negative relationship between muscle area and fibrosis, suggesting a possible relation also between sarcopenia and fibrosis. In fact muscle fibrosis has been shown to be related to a decrease in muscle strength and elasticity

as well as to reduced blood supply of muscle fibers, further increasing muscle fiber atrophy (Huard et al., 2002; Jarvinen et al., 2002).

Interestingly, we also observed a significant association between MS and MF and BMI, and in particular, between MS and MF and indices of fat distribution. These findings are in line with recent observations of higher degree of fibrosis and type VI collagen deposition in adipose tissue of obese subjects (Spencer et al., 2010). Compared with adipose tissue from lean subjects, adipose tissue from obese subjects contained increased areas of fibrosis, which correlated inversely with insulin sensitivity and positively with macrophage number (Spencer et al., 2010).

The mechanisms impairing muscle quality and leading to muscle wasting are still incompletely known (Janssen et al., 2010; Glass et al., 2010). Only a few studies were specifically designed in humans to investigate the molecular mechanisms contributing to sarcopenia (Leger et al., 2008). The pathways involved in this impaired muscle regeneration and enhanced fibrotic response typical of the aged muscle are still incompletely known (Brack et al., 2007; Graham et al., 2010; Trenz et al., 2010; Zhu et al., 2007). Myostatin, first identified as a negative regulator of muscular growth and regeneration, has been also implicated in the formation of fibrosis in skeletal muscle (Zhu et al., 2007). Myostatin mRNA and protein levels have been shown to be significantly increased in biopsies of vastus lateralis of elderly compared to young subjects (Leger et al., 2008). In the present study we observed a trend toward higher levels of myostatin expression in muscles of subjects with higher values of MQI and higher degrees of MF and MS. Confirming previous studies (Leger et al., 2008), we also described higher levels of mRNA of SOCS-3 and SREBP-1, in muscles with a higher substitution with fat and connective tissue. SOCS-3 is known to inhibit the activation of JAK/STAT pathway causing perturbations in GH signaling and therefore inhibiting muscle hypertrophy in favor of atrophy signaling cascade (Hansen et al., 1999); SOCS-3 is also implicated in triglyceride storage via its activation of SREBP-1 in different tissues (Ueki et al., 2005).

Finally in our study a worse muscle quality and a higher percentage of MF is related with higher levels of expression of IL-6 in SAT near the muscle, suggesting a cross-talk between the muscle and fat compartment in determining muscle function and quality.

Some limitations of the present study should be acknowledged. First, it is complex to study a histological phenomenon, like MF and MS, in a quantitative way; we tried to balance this intrinsic limit of our study using mean values of at least 2 readings of the same operator for each of the six sections analyzed with the image analysis software. Second, the design of the study allows us only to describe associations and not cause-effect relationships. Finally we acknowledge that the small sample size confers low statistical power, and these preliminary results should be confirmed in further studies.

In conclusion these preliminary data seems to show that MS and MF are related phenomena. In particular MF is related to muscle size, total and visceral adiposity, circulating leptin and hs-CRP. MS even in this small sample, confirming previous results, is associated with adiposity, visceral fat, leptin levels, insulin resistance and inflammation in the SAT. MS and

MF, even though different in their main determinant and pathogenesis, together concur to alter muscle quality and both should be considered in further studies on sarcopenia, sarcopenic obesity and muscle aging..

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Fig. 1.

Sections of PM biopsies in patients undergoing elective vertebral surgery with different degrees of MF and MS and MQI. Sections of PM biopsies stained with hematoxylin and eosin for histological and morphometric analysis: MF (*) and MS (§) area were measured using an image analysis software. MQI was calculated as mean percentage of MF and MS area (in three sections)/2. (A) Patient with MQI < 508 percentile (age 80 years; BMI 26 kg/m²). (B) Patient with MQI > 508 percentile (age 79 years; BMI 35.2 kg/m²).

Fig 2.
Comparison between different genes expression in subcutaneous (SAT) biopsies of patient with different degrees of MF and MS, evaluated by MQI.

Fig 3.

Comparison between different genes expression in paraspinous (PM) biopsies of patient with different degrees of MF and MS, evaluated by MQI. SOCS-3, suppressor of cytokine signaling-3; MYOST, myostatin; SREBP-a, sterol regulatory element binding protein-1; CEBP-a, CCAAT/enhancer-binding protein; MEF2, myocyte enhancing factor 2; IGF-1, insulin like growth factor 1; MURF-1, muscle ring finger-1; MYOD, myogenic regulatory factor D.

Table 1

Characteristics of study sample

Variable	Men (n=16)
Age (years)	70.3 ± 7.4
BMI (kg/m ²)	30.4 ± 4.8
Waist circumference (cm)	104.8 ± 11.1
FM (kg)	25.6 ± 6.9
FM (%)	28.9 ± 4.0
LM (kg)	58.9 ± 8.4
Paraspinous muscle area (cm ²)	431.4 ± 160.5
IMAT (cm ²)	1384.9 ± 707.5
Leptin (ng/ml)	11.5 ± 8.2
Adiponectin (µg/ml)	6.0 ± 2.0
Hs-CRP (mg/ml)	6.6 ± 6.5
IL-6 (pg/ml)	22.9 ± 12.0
Glucose (mmol/L)	5.6 ± 0.8
Insulin (mU/L)	5.7 ± 1.4
HOMA	2.4 ± 1.5

Data are presented as mean ± standard deviations.

BMI = body mass index; FM = Fat Mass; LM = Lean Mass; IMAT = intermuscular adipose tissue; hs-CRP = high sensitivity C reactive protein; HOMA= homeostasis model assessment of insulin resistance.

Table 2

Simple correlations between muscle quality, and anthropometric, body composition variables, intermuscular adipose tissue, adipokines, inflammation and insulin resistance in healthy elderly men (n=11).

	Myofibrosis (r)	Myosteatorsis (r)
BMI	0.602 *	0.717 *
Waist circumference	0.763 **	0.713 *
Weight	0.497	0.646 *
FM #	0.662 *	0.865 ***
FM% #	0.681 *	0.872 ***
LM #	0.123	0.106
App LM #	-0.071	-0.124
IMAT §	0.620 *	0.849 ***
Paraspinous muscle area §	-0.700 *	-0.341
Leptin	0.809 **	0.864 ***
Adiponectin	-0.327	-0.336
IL-6	-0.269	-0.187
hs-CRP	0.591 *	0.418
Glucose	0.280	0.358
Insulin	0.164	0.436
HOMA index	0.218	0.455

BMI = Body Mass Index; FM= Fat Mass; LM = Lean Mass; App LM = Appendicular Lean Mass; IMAT = Intermuscular Adipose Tissue. hs-CRP = high sensitivity C reactive protein; HOMA index = homeostasis model assessment of insulin resistance.

measured by Dual Energy X-ray Absorptiometry;

§ measured by MRI

* = p < 0,05;

** = p < 0,01;

*** = p < 0,001

Table 3

Backward regression analyses where Myofibrosis or Myosteatosis are the dependent variables and age, waist circumference, paraspinous muscle area, leptin, hs-CRP and HOMA index the independent variables in healthy elderly men (n=11).

Myofibrosis	R ²	β Coefficient	p	Myosteatosis	R ²	β Coefficient	p
1. Age	.796	-.066	.736	1. Age	.709	-.099	.675
BMI		-.048	.903	BMI		-.218	.647
Waist circumference		.590	.172	Waist circumference		.475	.316
Leptin		.361	.511	Leptin		.945	.202
Muscle area		-.580	.046	Muscle area		.030	.895
HOMA index		-.206	.535	HOMA index		.031	.935
Hs-CRP		-.127	.648	Hs-CRP		-.254	.459
2. Age	.846	-.064	.702	2. Age	.781	-.100	.619
Waist circumference		.561	.058	BMI		-.266	.566
Leptin		.328	.416	Waist circumference		.465	.231
Muscle area		-.589	.013	Leptin		.988	.026
HOMA index		-.197	.474	Muscle area		.030	.877
Hs-CRP		-.114	.601	Hs-CRP		-.268	.291
3. Waist circumference	.872	.537	.035	3. Age	.824	-.099	.578
Leptin		.336	.356	BMI		-.201	.525
Muscle area		-.589	.006	Waist circumference		.452	.175
HOMA index		-.194	.432	Leptin		.966	.008
Hs-CRP		-.141	.456	Hs-CRP		-.267	.235
4. Waist circumference	.879	.576	.017	4. BMI	.843	-.185	.529
Leptin		.164	.529	Waist circumference		.400	.171
Muscle area		-.572	.003	Leptin		.977	.004
HOMA index		-.097	.622	Hs-CRP		-.306	.133
5. Waist circumference	.892	.630	.002	5. Waist circumference	.855	.266	.128
Leptin		.064	.670	Leptin		.906	.001
Muscle area		-.570	.002	Hs-CRP		-.260	.139
6. Waist circumference	.903	.667	.000	6. Waist circumference	.823	.244	.189

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Myofibrosis	R ²	β Coefficient	p	Myosteatosis	R ²	β Coefficient	p
Muscle area		-.591	.000	Leptin		.755	.002
				7. Leptin	.802	.906	.000

BMI = body mass index; hs-PCR = high sensitivity C reactive protein; HOMA= homeostasis model assessment of insulin resistance.