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Association between Skeletal Muscle Atrophy and Denervation in Older Hip Fracture Patients with Sarcopenia

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Fabiana Tanganelli

To my grandparents,

who would be proud of this aim from their brighter silence;

to myself,

and to the many goals to be achieved.

Fabiana Tanganelli

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A Acknowledgments

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B Summary

Fracture of the proximal femur in old age is one of the conditions with the greatest prognostic impact. Recent studies indicate that 1.6 million elderly people in the world, sustain a femur fracture. Due to the progressive aging of the population in industrialized countries, it is expected that the incidence of femoral fractures will reach 2.6 million of new cases by 2025 and 4.5 million in 2050. These epidemiological data have a considerable economic and health impact, due to the enormous welfare and indirect costs associated with the frequent development of disabilities and other self-sufficiency problems. Consequently, the development of new strategies to improve survival and the functional recovery of this population represents one of the main biomedical objectives in the geriatric field research. Among the determinants of the quoad vitam and functional prognosis of elderly patients with hip fracture, sarcopenia has been shown to be one of the potential factors susceptible to intervention. This condition, that is an age-related loss of muscle mass and strength, is object of particular clinical and research interest.

During the aging, there is a preferential loss of motoneurons involved in type 2 muscle fiber innervation. In that case, the denervated fiber are reinnervated in the process called motor unit remodeling with consequently co-expression of myosin heavy chain (MHC) fast and slow in the same fiber. If the denervation is more preponderant than reinnervation, it can lead to atrophy and sarcopenia. The aim of this work was, therefore, to find histomorphological markers of sarcopenia; to study the denervation and reinnervation process from histological point of view in sarcopenic cohort, using specific biomarkers, to find an association between denervation and loss of muscle mass, quality and strength.

The enrollment of study participants was conducted from November 2017 to March 2019. They have been subjects aged > 70 years of both sexes with hip fracture. The exclusion criteria were: age < 70 years, specific neuromuscular diseases (myasthenia gravis, muscular dystrophy, ALS, polio), severe dementia, chronic inflammatory disease (e.g. Crohn's dis-ease, ulcerative colitis, rheumatoid arthritis), systemic corticosteroid therapy, and cancer therapy in the last 5 years. The presence of sarcopenia was established on the basis of the criteria of the European Working Group on Sarcopenia in Older People 2 (EWGSOP2). Lean body mass measurement was performed through bioimpedance analysis (BIA), while muscle strength was measured by manometry.

Our experiments focused on the histological analysis of biopsies in muscle tissue samples taken during the femoral fracture stabilization surgery. In particular, we measured the diameters of type 1 and type 2 fibers, assessed the coexpression of the two myosin isoforms (MHC slow and MHC fast) capable of marking them; analyzed the presence of positive fibers at nMHC, NCAM, pyknotic nuclei as denervation markers and PAX7 to study also the regenerative capacity of the fibers. In particular, we determined the protein expression levels of MHC fast and slow, the coexpression of both the fast and slow isoforms, nMHC, NCAM, pyknotic nuclei and Pax 7.

The results of the present study show that sarcopenia was identified in 5 patients (3 women and 2 men), probable sarcopenia in 11 patients (4 women and 7 men). Significant differences were found among all three groups for fiber type-2 in men with non- sarcopenia (59 µm), probable sarcopenia (49µm) and sarcopenia (34 µm). In women, significant differences were revealed in fiber type-1 between non-sarcopenia (58 µm) and sarcopenia (69 µm). Only 1-3% mixed fiber types were found in sarcopenic patients, indicating a final

stage where reinnervation is not possible to occur anymore. Muscle fiber type-2 atrophy in men seems to be a histological marker for sarcopenia.

With regard of regeneration, an association between SMI and PAX7 was found (r=0.439 *p*=0.012) in all population and in women the loss of regeneration is correlated with larger motor unit size (MUSIX) (r= 0.523 p=0.031). Considering the motor neuron, a correlation between MUNIX and NCAM (r = 0.793 *p*= 0.003) in men was detected. An inverse correlation between nMHC and age (r=-0.546 *p*=0.04) was found in men and between NCAM and age (r= -0.575 *p*=0.032; r= -0.522 *p*= 0.026) was found in men and women respectively. Denervation appears to be related to a low number of motor unit and to age, especially in men, but not to sarcopenia in our cohort.

The data obtained from this study are the first to be produced on the subject and therefore represent the basis for future and more complex investigations aiming to improve knowledge on the pathogenesis of sarcopenia.

INTRODUCTION

1. Sarcopenia

During the aging the most predominant changes are a reduction of mass and increase in fat mass (Tzankoff & Norris, 1978), with functional and metabolic consequences. It was not until 1989 that the word Sarcopenia was first introduced by Rosenberg to express association with frailty, disability and mortality, and to describe changes in body composition among older people (IH, 2010). Sarcopenia derives from the Greek: *sarx* which means meat and *penia* which means loss (Rosenberg, 1997).

A general decline of skeletal muscle mass starting between 30 and 40 years of age with approximately 3–8% per decade, and accelerates after age 60 (Jan Lexell et al., 1988).

After Rosenberg's definition, sarcopenia was defined as a disease characterized by the progressive general decline in muscle mass age dependent (Roubenoff & Hughes, 2000). Anyhow, the entire scientific community has not been totally in agreement on this definition, and this is the reason why there have been evolutions of the formulation of the correct definition of sarcopenia over the years. It was in 2010 that the European working group on sarcopenia in the elderly (EWGSOP) established criteria for the diagnosis of sarcopenia (A. J. Cruz-Jentoft et al., 2010). This definition had introduced muscle function, which is why it represented an important change, as until then sarcopenia was only associated with decreased muscle mass.

However, 10 years later, the working group met again (EWGSOP2) in order to update the algorithm based on the scientific evidence accumulated up to then. A new definition of sarcopenia has been introduced as a progressive and generalized skeletal muscle disease that has an impact on clinical out-

comes including falls, fractures, disabilities (Alfonso J. Cruz-Jentoft et al., 2019).

Histologically, sarcopenia is feature by atrophy of muscle fibers, with a preferential loss of type-2 (fast-twitch) fibers (Jan Lexell et al., 1988), which are associated with multiple factors (A. J. Cruz-Jentoft et al., 2010), such as low grade chronic inflammation (J.A. et al., 2014), insulin (Alemán-Mateo et al., 2014) and anabolic resistance (Burd et al., 2013; Cuthbertson et al., 2005), hormonal changes (Anderson et al., 2017; Messier et al., 2011), mitochondrial dysfunction (M.T. et al., 2013), oxidative stress (Joseph et al., 2016), malnutrition (Cruz et al., 2017), inactivity (Marzetti et al., 2017) and motor neuron loss (Drey et al., 2014) contribute to progressive and adverse changes in aging muscle.

2. Skeletal muscle fiber

Skeletal muscle fibers are composed of the myofibrillar proteins myosin (the thick filament) and actin (the thin filament). The set of these functional units is defined sarcopero. In order for muscle twitch to occur, the 2 myofibrillar proteins interact among them.

The myosin molecule is composed by a heterohexamer consisting of 2 heavy chains and 4 light chains (2 regulatory and 2 alkali) that interact non-covalently with each other. In particular, we find two heavy myosin chains (MHCs) formed by an α-helix tail and a globular head which constitutes the motor domain as it has ATPase activity and is able to bind to the actin monomers of the then filaments. The weight of each heavy chain is around 220 kD. In correspondence of the region of passage between head and tail (some-

times referred to as neck) four light myosin chains are associated (myosin light chains - MLCs) which can be conventionally grouped two by two(Reggiani et al., 2000).

MHCI to label fiber type 1, MHCIIa fiber type 2a, and MHCIIx/d to label fiber type 2b (formerly erroneously identified as MHCIIb). The myosin heavy chain isoforms have been used to classify fiber types and MHCIIb is not expressed in human fibers (Schiaffino & Reggiani, 2011). The difference between fiber type 1 and type 2 fiber also lies in the metabolism, aerobic of the fiber type 1 that produces ATP necessary for muscle contraction through aerobic glycolysis and oxidative phosphorylation, and the fiber type 2 characterized by a metabolism anaerobic with high ATPase activity, they produce ATP through anaerobic glycolysis (Scott et al., 2001).

In humans and in other mammals, fibers type 2 are more involved than fibers type 1 in the damage induced by twitch (Schiaffino & Reggiani, 2011).

3. Motor Unit

The causes of sarcopenia are still under study. For voluntary movement, efficient communication between the nervous and muscular systems is required. This triggers the excitation of the upper motor neurons that transmit the action potential to the lower motor neurons within the posterior area of the spinal cord. The electrical impulse then spreads from the cell body of a motor neuron to the neuromuscular junction (NMJ), a specialized synapse between the neuron and the muscle. After this, acetylcholine transmits the neuronal action potential arriving at the sarcolemma where the depolarization of the muscle fiber is triggered and the contraction begins (Rygiel et al., 2016).

During the aging motoneurons driving large, fast motor units (MUs) seem differentially susceptible to premature death, to unknown causes

(Campbell et al., 1973; Jan Lexell, 1997; McNeil et al., 2005). Among all the factors, as previously reported by Drey et al. 2014, loss of motor unit is also associated with sarcopenia. Then, when the connection between the nervous and muscular systems fails the consequence are muscle atrophy, and loss of muscle strength and power (Scelsi et al., 1980). Muscle fibers cannot remain denervated, so the denervated muscle fibers are reinnervated from neighboring fibers. This can cause two significant alterations in the aged neuromuscular system: the germination from other axons does not provide reinnervation, then the muscle fibers affected eventually die and cause contractile weakness age dependent; or denervation is followed by incomplete fiber recruitment, which may affect the overall distribution of the type of fiber in older muscles, which leads to fiber type shifting (Doran et al., 2009). The transition from fast to slow fiber is supported by the conversion from a bioenergetic process dominated by glycolysis to a greater oxidative metabolism in elderly muscles (Doran et al., 2007).

It has been proposed that an increase in MU size with decreasing numbers of neurons results in a declining ability to re-innervation (Siu & Gordon, 2003). If the capacity for neurons to reinnervate adjacent denervated muscles is retained with age, this implies the question of what alterations are causing the permanent denervation associated with atrophic fibers. One hypothesis is that absolute loss of α-MNs with age results in a decreased capacity for neuronal sprouting following denervation (Campbell et al. 1973; Rowan et al. 2012). The cause of α-MN death remains controversial despite many studies investigating age related changes in neuron cell body number within the lumbosacral portion of the spinal cord. An investigation from Tomlinson and Irving (1977) (Tomlinson & Irving, 1977) suggest that there are no significant chang-

es in cell number until age 60, after which a negative correlation between cell number and patient age was observed. By age 90, a 70% decrease in α-MNs.

4. Hip fracture, sarcopenia in elderly

Sarcopenia puts older people at high risk of falls and fractures (Landi et al., 2012). The prevalence of sarcopenia is between 2% and 37% in elderly living in community, which depends on the type of sarcopenia criteria used (Bijlsma et al., 2013; Shafiee et al., 2017). Evidence shows that treating and preventing sarcopenia is important in increasing muscle mass, strength and physical performance (Beaudart et al., 2017; Papa et al., 2017), although it has not yet been shown that this leads to a decrease in falls and fractures.

It has been estimated that about 30% of older people living in communities over 65 years fall at least once a year (Graafmans et al., 1996). This percentage increases by 10% in people over 80 years of age (Pohl et al., 2014). Hip fracture accounts for 28% of hospitalizations for falling in elderly patients (Hartholt et al., 2011). Falls and fall injuries can lead to disability (Hartholt et al., 2011), morbidity (Farahmand et al., 2005) and mortality (Tajeu et al., 2014).

1.6 million of elderly people worldwide have been shown to sustain femur fracture every year (Hung et al., 2012), with a mortality rate greater than 25% (Maggi et al., 2010). Due to the progressive aging of the population in industrialized countries, an incidence of 2.6 million new cases of femoral fracture and 4.5 million in 2050 is expected in the coming years (Cauley et al., 2014).

These numbers have a considerable impact on the economic health plan due to the enormous care and disability-related costs (Pike et al., 2010).

D OBJECTIVES

The aims of this project are:

1. to find histomorphological markers of sarcopenia;
2. to study the denervation and reinnervation process from histological point of view, using specific biomarker, to find an association between denervation and sarcopenia in our study cohort.

E MATERIALS AND METHODS

1. Materials

1.1 Lab equipment

1. Camera: UC90 camera (Olympus)

2. Centrifuges: Centrifuge 5417, 5417R (Eppendorf, Hamburg, Germany); Varifuge 3.0R (Heraeus, Buckinghamshire, UK)

3. -80°C Freezer: HERA freeze (Heraeus, Buckinghamshire, UK)

4. Microscope: CKX53 microscope (Olympus), BX 61 fluorescence microscope (Olympus)

5. pH-meter: HI9321 Microprocessor pH Meter (Hanna Instruments, Kehl am Rhein, Germany)

6. Pipetes: Pipetman, Gilson and Eppendorf (2 µl, 20 µl, 200 µl, 1000 µl)

1.2 Staining equipment

1.2.1 Primary Antibodies

1. Technical specifications of the primary antibodies used for immunohistochemistry:

	Manufacture and Catalogue number	Type	Species	Dilution used
Primary Antibody				
Anti-MHC slow	Sigma-Aldrich, M8421	Monoclonal	Mouse	1:3000
Anti-MHC fast	Sigma-Aldrich, M4276	Monoclonal	Mouse	1:3000

2. Technical specifications of the primary antibodies used for immunofluorescence:

	Manufacture and Catalogue number	Type	Species	Dilution used
Primary Antibody				
Anti-MHC slow	Sigma-Aldrich, M8421	Monoclonal	Mouse	1:600
Anti-MHC fast	Abcam, ab91506	Polyclonal	Rabbit	1:500
Anti-nMHC	Novocastra, NCL-MHCn	Monoclonal	Mouse	1:20
Anti-Desmin	Cell Signaling, D93F5	Polyclonal	Rabbit	1:50
Anti-NCAM	Millipore Sigma, ab5032	Polyclonal	Rabbit	1:100
Anti-PAX 7	Abcam, ab34360	Polyclonal	Rabbit	1:50
Anti-β-Dystroglycan	LSBio, LS-B7929	Monoclonal	Mouse	1:50

Abbreviations: MHC slow: myosin heavy chain slow; MHC fast: myosin heavy chain fast; nMHC: neonathal myosin heavy chain; NCAM: neural cell adhesion molecule; PAX 7: paired box protein.

1.2.2. Secondary Antibodies

The secondary antibodies used for immunohistochemistry were Discovery™ Universal Secondary Antibody Secondary antibody for use with Discovery™ Detection kits on the Ventana Discovery™ Staining Platform.

Technical specifications of the secondary antibodies used for immunofluorescence:

	Manufacture and Catalogue number	Conjugate	Host	Dilution used
Secondary Antibody				
Anti-mouse IgG	Invitrogen	Alexa Fluor 595	Monkey	1:500
Anti-rabbit IgG	Invitrogen	Alexa Fluor 488	Monkey	1:500
Anti-mouse IgG	Invitrogen	Alexa Fluor 488	Monkey	1:500
Anti-rabbit IgG	Invitrogen	Alexa Fluor 595	Monkey	1:500

1.3 Software using

Ventana Discovery™ Staining Platform, Cell Sens, SPSS Statistics 25, Image studio, Illustrator CS2 (Adobe); ImageJ (national institute of health, USA).

1.4 Staining solutions

1. Harris hematoxylin solution

Filter the Roth X903.2 / 1l before each use

2. Eosin solution

Downtown pharmacy Eosin 5% aqueous No.45380 = dilute 1: 3 with Bidest + approximately 5 drops of acetic acid.

3. Tris- buffered- saline (TBS) buffer, 10 x

0.2 M Tris-HCl, 0.8% NaCl in 1l H_2O pH 7.6

4. TBS_t 1X

for $TBS_{t(tween)}$ add Tween 20 (Sigma) to 0.1%.

For 1 l use 100 ml 10x TBS and 1 ml Tween 20 (Sigma).

2 Methods

2.1 Participants

We recruited patients with proximal hip fracture of the femur aged over 70 years undergoing surgery. The exclusion criteria were: age < 70 years, specific neuromuscular diseases (myasthenia gravis, muscular dystrophy, ALS, polio), severe dementia, chronic inflammatory disease (e.g. Crohn's disease, ulcerative colitis, rheumatoid arthritis), systemic corticosteroid therapy, and cancer therapy in the last 5 years. Patient recruitment started in November 2017 and ended in March 2019.

All participants provided written informed consent before enrolment. The study protocol was approved by the Ethics Committee of LMU Munich (IRB-No. 328-15). During hip fracture surgery, an open biopsy of the vastus lateralis muscle was performed.

2.2. Diagnosis of Sarcopenia

According to the European Working Group on Sarcopenia in Older People 2 (EWGSOP2) criteria we defined sarcopenia in our study cohort (Alfonso J. Cruz-Jentoft et al., 2019). After surgery bioelectrical impedance analysis (BIA) was performed in standard condition, with the patient in a supine position and surface electrodes placed on the wrist and ankle contralateral to the side of the fracture. Appendicular lean mass (aLM) was estimated using the equation developed by Sergi et al. (2015). The Skeletal muscle index [SMI, (kg/m2)] was obtained by dividing aLM by squared body height. Low SMI was defined below 7 kg/m^2 and 5.5 kg/m^2, in men and women, respectively. Handgrip strength was assessed with a Saehan DHD-1 Digital Hand Dynamometer. The maximal value of three consecutive measurements of both hands was used for the analysis. The cut off for the handgrip strength was defined below 27 kg in men and 16 kg in women (Alfonso J. Cruz-Jentoft et al., 2019).

All measurements for diagnosis of sarcopenia were carried out by the medical students.

2.3 Degree of sarcopenia

For a metric measure of the degree of sarcopenia, a sarcopenia Z-score was calculated based on EWGSOP2 criteria, using the individual participant's data and the standard deviation (SD) obtained from the baseline data of the our study cohort (Kemmler et al., 2016; Figure 1).

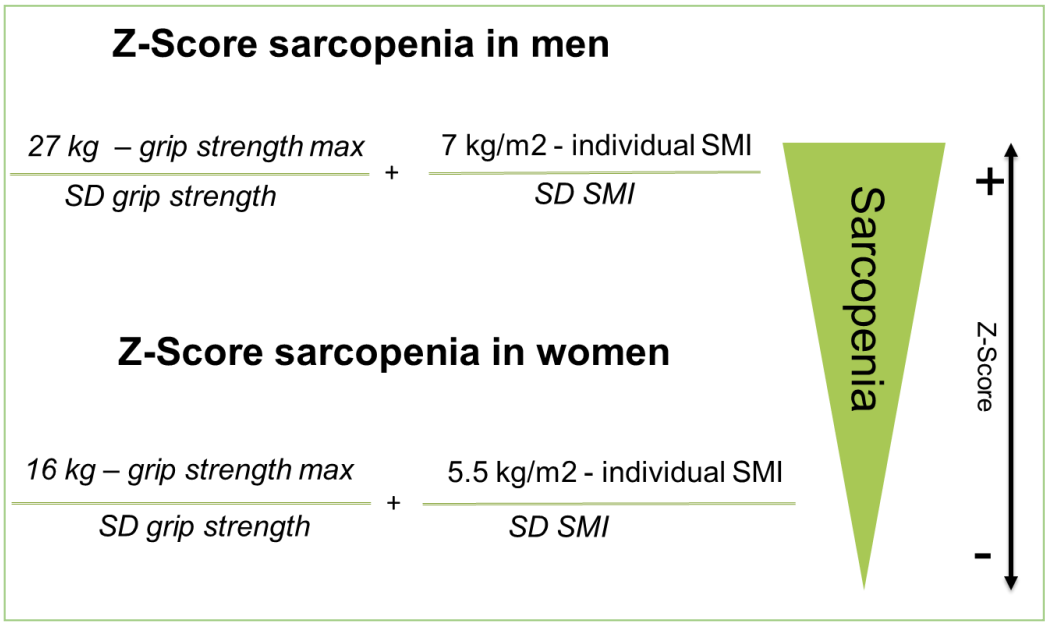

Figure 1. Representation of Z-Score calculation and the comparison with sarcopenia degree.

Negative value of Z-scores were favorable, and the reduction of Z-Score decreases sarcopenia risk.

2.4 Munix technique

In order to study the neurogenic aspect of sarcopenia Motor Unit Number Index (MUNIX) was performed, and even in this case like for sarcopenia's diagnosis, measurements were taken by medical students. MUNIX is electromyographic method used for assessing the number and size [motor unit size index (MUSIX)] of MUs (motor units) using the compound muscle action potential (CMAP) and the surface electromyographic interference pattern (SIP) (Neuwirth et al., 2010).

MUNIX is a technique divided in 3 steps. First, CMAP is calculated using three supramaximal stimulations of ulnar nerve to obtain the highest possible CMAP amplitude used to calculate its amplitude, area, and power. The SIPs are recorded in the second step to the patient with isometric contraction at

varying levels of effort. Once obtained, the CMAP and SIP signals are inserted into the analysis software which calculates the area and power of the signals, to obtain the MUNIX. MUSIX value is obtained by dividing CMAP amplitude and MUNIX (Drey et al., 2014).

2.5 Staining

Skeletal muscle tissue blocks were cut to 10-µm tissue sections on a cryostat (HM505E; Microm, Walldorf, Germany) at -26°C and mounted on glass slides (Double frosted microscope slides; Fisher Scientific), air dried for 2h and stored at -80°C.

2.5.1 Immunohistochemistry staining

The most common stain used in routine is hematoxylin and eosin (H&E, **figure 2**). It is a bicromatic labelling that is based on the different pH value of the various tissues and organelles that make up the cell. The nucleus and the various acid components of the cytoplasm (ribosomes, acid secretions) are colored in purple by hematoxylin, which is a basic dye, while the cytoplasm and the various basic tissues (muscle, connective, bone) are colored in pink, more or less intense, from an acid mixture of eosin. This protocol describes H&E staining in vastus lateralis muscle.

The slides were put 10 minutes in 4% formalin solution, 5 minutes in the tap water and rinsed in distilled water. 5 minutes of Harris rinse in distilled water, 7 minutes of cold tap water, rinse in distilled water; 5 minutes of eosin. Wash one or two times in distilled water, 70% alcohol, 96% alcohol, 96% alcohol, 100% alcohol and 100% xylene alcohol to cover the slide.

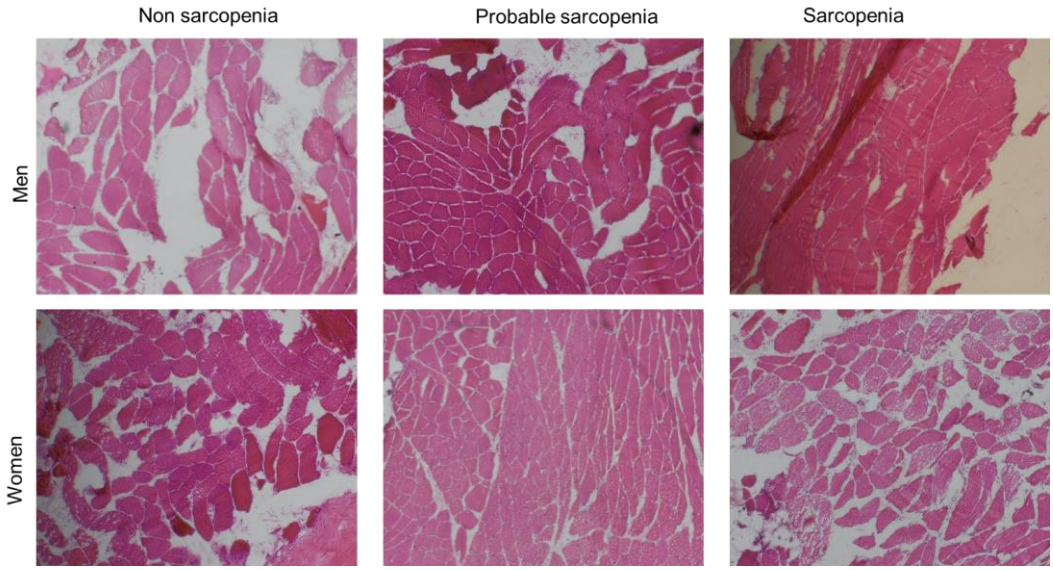

Figure 2. Transverse sections of muscle fibers labelled with H&E.

In order to study muscle atrophy, it was necessary to distinguish fiber type 1 and type 2, responsible respectively for the slow and fast contraction of the muscle fiber. For this reason, it was decided to use, at first, the coloring of routine with anti MHC slow isoform most expressed in type 1 fiber; and anti MHC fast myosin isoform more expressed in type 2 fiber.

These two distinct stains were performed using Discovery™ Detection kits on the Ventana Dis-covery™ Staining Platform. The secondary antibodies used were Discovery™ Universal Secondary Antibody Secondary antibody.

2.5.2 Immunofluorescence staining

Muscle cross-sections were used for the co-staining using, in the same slice, two different primary antibodies from different species (see Material). We have combined ABs anti myosin heavy chain (MHC) fast and slow in one slice, in order to study the co-expression of myosin in the same fiber (mixed fiber); in addiction other labellings were made to evaluate the expression of denerva-tion markers: anti Pax7 and anti β-dystroglycan; anti neonatal MHC and anti

desmin, anti NCAM and anti MHC slow. Slides were first removed from -80°C and allowed to air dry for 15 minutes at room temperature. Sections were hydrated with TBS_{1x} three time every 5 minutes, incubated for 1h at room temperature with the Blocking solution ($TBS_{t\ 0,1\%}$ and Gelatin Fish 0,9%) and after that with the following primary antibodies over night: monoclonal mouse MHC slow , polyclonal rabbit anti-MHC fast, polyclonal rabbit anti PAX7, monoclonal mouse anti β-dystroglycan, monoclonal mouse anti-nMHC and polyclonal rabbit anti-desmin, polyclonal rabbit anti NACM. After incubation with primary ABs, tissue sections were washed three times with $TBS_{t\ 0,1\%}$ and incubated for 1h with the second ABs: anti-mouse, anti-rabbit.

Sections were washed two times with $TBS_{t\ 0,1\%}$ and two times with TBS_{1x}, cover with mounting medium for fluorescence with DAPI (Vertor Laboratories, Inc. Burlingame, CA 94010).

2.5.3 Measures

In order to study the degree of skeletal muscle atrophy we decided, as first step, to measure the diameter of fiber type 1 and type 2. We have used the measurement of the "lesser diameter" measurement as it can happen that the fiber is distorted, in some cases it is cut obliquely and its shape appears oval. Unless the bottom diameter is measured, it will result in an erroneously large measurement. Measuring the bottom diameter will avoid mistakenly large measurement.

A mean of 250 muscle fibers per biopsy have been evaluated, comparing fibers type 1 and type 2 for relative prevalence. We considered as atrophic fibers those have diameter lower than 30 and 40 μm, which is the minimum value of the normal range for women and for men (Dubowitz V, Sewry CA, Oldfors A Lane R, 2013).

In addition, in an area of approximately 100 fibers we calculated how much (as a percentage) were type 1 and type 2 and which was stirring, which means co-expression of MHC fast and slow in the same fiber. This is because the mixed fiber is a typical marker for the reinnervation (Andersen, 2003).

In order to express the number and the extent of excessively small and large fibers in the biopsies, respectively, atrophy factor (AF) and hypertrophy factor (HF) were calculated (Dubowitz V, Sewry CA, Oldfors A Lane R, 2013).

To obtain the AF, in women the number of muscle fibers with diameter of between 29.9 and 20 μm, between 19.9 and 10, and < 10 μm were multiplied by 1,2 and 3, respectively, and the sum of these products was divided by the total number of fibers counted, and multiplied by 1000. In men the atrophy factor was calculated considering in addiction also the range between 39.9 and 30 μm, and multiplied the results of the 4 ranges by 1,2,3 and 4. The HF was similarly obtained by multiplying the numbers of fibers larger than 80 μm and 70 μm, respectively in the men and in women (Dubowitz V, Sewry CA, Oldfors A Lane R, 2013). Data obtained with normal vastus lateralis muscle using this method show AF FT1 150 and 100, AF FT2 150 and 200, HF FT1 150 and 400, HF FT2 400 and 150, in men and in women respectively (Dubowitz V, Sewry CA, Oldfors A Lane R, 2013).

The percentage of type 1, 2 and mixed fiber, the pyknotic nuclei seen with HE stains, the PAX7, NCAM and nMHC positive fibers were counted with ImageJ software (national institute of health, USA).

2.6 Statistical Analysis

Statistical analysis was performed using PASW 21.0 (IBM-SPSS Inc., Chicago, II, USA). The characteristics of study participants were divided by sex. Differences between the two groups were assessed by the Student's t-

test, and ANOVA test were used to study the differences among all three groups. Multiple linear regression analysis was used to describe associations between clinical data (Z-Score, SMI, handgrip strength, MUNIX, MUSIX) and histological characteristics. For all tests the statistical significance was set at $p < 0.05$.

F RESULTS

1. Characteristics of study cohort

We recruited a total number of 37 patients aged over 70 years of both sexes. Among these we could analyze histological data for 32 patients. The median age of the patients used for this investigation was 83.3 years (**Table 1**); the mean age between men and women was similar and did not differ significantly. Significant sex differences were observed for handgrip strength and skeletal muscle index (**Table 1**).

Table 1. Characteristics of the study cohort and comparison between men and women group

	Total sample median (n=32)	Total sample mean (n=32)	Men (n=14)	Women (n=18)	p-value
Demographic and anthropometric characteristics					
Age [years]	83.3	82.2 ±6.2	82.7 ± 6.0	81.9 ± 6.5	0.728
BMI [kg/m²]	25.0	24.6 ±4.2	24.2 ± 3.9	25.0 ± 4.5	0.595
Handgrip strength [kg]	20.8	22.6 ± 8.8	27.0 ± 9.1	19.1 ± 6.4	**0.009**
SMI [kg/m²]	7.1	6.9 ± 1.2	7.4 ± 1.1	6.4 ± 1.0	**0.018**
Z-score sarcopenia	-0.7	-0.9 ± 1.7	-0.37 ± 1.8	-1.3 ± 1.7	0.125
MUNIX	79	92.7 ± 43.5	84.5 ± 42.0	98.5 ± 44.8	0.395
MUSIX	79	78.7 ± 37.1	96.7 ± 51.8	75.9 ± 19.3	0.140
Histological characteristics					
Fiber type 1 [µm]	58.5	60.3 ± 10.5	60.5 ± 13.2	60.1 ± 8.3	0.902
Fiber type 2 [µm]	44.6	46.1 ± 9.7	50.3 ± 10.9	42.8 ± 7.3	**0.027**
Fiber type 1 [%]	57,2	59.5 ± 18.6	60.4 ± 17.6	58.8 ± 19.8	0.813
Fiber type 2[%]	30.0	30.8 ± 14.5	31.4 ± 15.7	30.3 ± 13.9	0.832
Mixed fiber type [%]	5.1	9.6 ± 11.3	8.1 ± 9.7	10.9 ± 12.6	0.508
nMHC+ [%]	1.0	1.6 ± 2.3	2.4 ± 2.9	1.0 ± 1.5	0.086
NCAM+ [%]	1.0	1.8 ± 2.3	1.2 ± 1.6	2.3 ± 2.7	0.199
Pyknotic Nuclei [%]	1.7	2.0 ± 2.1	1.6 ± 1.1	2.3 ± 2.6	0.332
PAX 7+ [%]	0.9	0.8 ± 0.8	1.1 ± 1.0	0.6 ± 0.7	0.099
Hypertrophy factor fiber type 1	192.0	371.6 ± 404.3	278.9 ± 451.2	443.6 ± 360.2	0.259
Hypertrophy factor fiber type 2	18.2	77.5 ± 179.0	115.8 ± 261.8	47.0 ± 61.8	0.288
Atrophy factor fiber type 1	10.7	71.5 ± 124.1	147.6 ± 158.4	12.3 ± 20.7	**0.001**
Atrophy factor fiber type 2	191.83	256.2 ± 283.8	374.9 ± 372.7	163.8 ± 141.5	**0.035**

p values between groups were calculated using two-sample-T-test

Based on the criteria of the European Working Group on Sarcopenia in Older People 2 (EWGSOP2) (Alfonso J. Cruz-Jentoft et al., 2019) we divided our patients in three groups:

(I) Non-sarcopenia - SMI > 7 kg/m2 (♂) / 5.5 kg/m2 (♀) and hand grip strength > 27 kg (♂) / 16 kg (♀)

(II) Probable-sarcopenia - SMI > 7 kg/m2 (♂) / 5.5 kg/m2 (♀) and hand grip strength < 27 kg (♂) / 16 kg (♀)

(III) Sarcopenia - SMI < 7 kg/m2 (♂) / 5.5 kg/m2 (♀) and hand grip strength < 27 kg (♂) / 16 kg (♀)

According to this classification, our cohort was composed, in men, by 5 patients with non-sarcopenia, 7 with probable sarcopenia, and 2 with sarcopenia. For the women we found 11 patients to be non-sarcopenic, 4 with probable-sarcopenia and 3 with sarcopenia (Table 2). The BMI in both men and women with sarcopenia is decreased, compared with other groups. It is appropriate to report that only men with sarcopenia have this value below the normal range 18.5- 24.9 kg/m^2 (Dowsey et al., 2018).

Table 2. Classification of our patient cohort for sarcopenia (mean values and standard deviation)

	Men			Women		
	Non sarcopenia (n = 5)	Probable sarcopenia (n = 7)	Sarcopenia (n = 2)	Non sarcopenia (n = 11)	Probable sarcopenia (n = 4)	Sarcopenia (n = 3)
Age (y)	82.1 ± 5.3	82.0 ± 6.5	86.3 ± 0.7	81.2 ± 7.0	85.5 ± 3.3	79.5 ± 4.7
BMI [kg/m²]	25.1 ± 1.1	25.4 ± 2.5	17.5 ± 4.9	27.0 ± 3.3	23.8 ± 4.3	19.2 ± 1.2
Handgrip str. [kg]	37.6 ± 5.2	22.5 ± 3.6	18.2 ± 3.0	22.6 ± 10.1	13.3 ± 3.1	13.4 ± 1.9
SMI [kg/m²]	8.0 ± 1.0	7.5 ± 0.5	5.9 ± 1.1	6.6 ± 0.8	7.3 ± 0.9	5.0 ± 0.4
Z-score	-2.0 ± 1.4	0.0 ± 0.7	2.3 ± 1.8	-2.2 ± 1.4	- 0.6 ±0.8	0.9 ± 0.4
MUNIX	79.1 ± 39.3	91.9 ± 52.1	71.8 ± 21.9	111.1 ± 45.4	62.4 ± 10.8	88.7 ± 52.0
MUSIX [µV]	134.3 ± 75.9	77.9 ± 27.3	78.0 ± 0.9	74.1 ± 17.3	91.66 ± 25.5	66.7 ± 18.0

2. Size and number of muscle fibers

The results of all stainings are summarized in **table 3** divided by sex, where we found a significant difference in fiber type 2 diameter (p= 0.027), AF fiber type 1 (p= 0.001), and AF fiber type 2 (p= 0.035), and in **table 3** divided by sex and groups. Decrease in fiber diameter during aging is a well-known phenomenon.

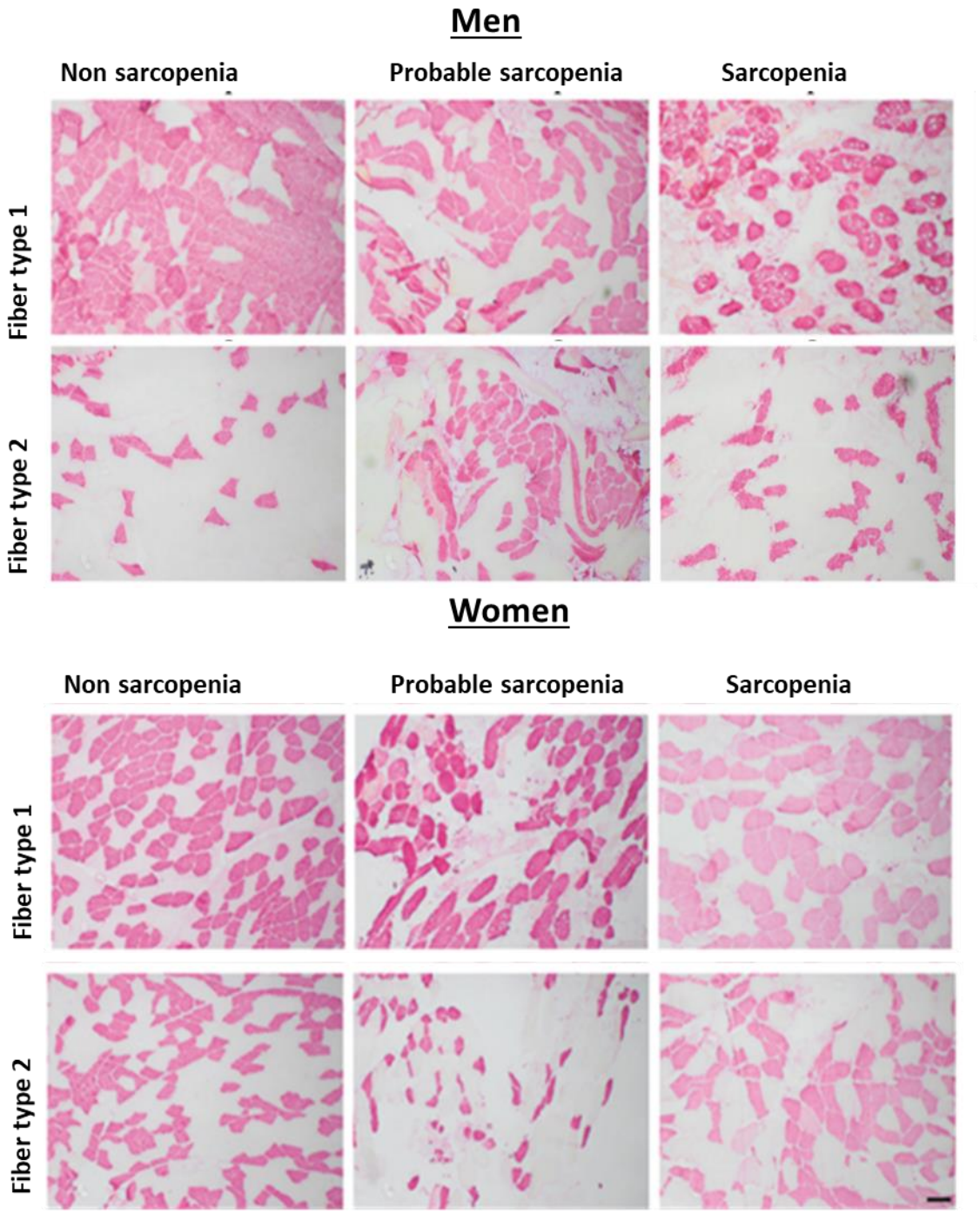

Figure 3. Immunohistochemical staining for type-1 and type-2 fibers, divided by sex and clinical classifications. Scale bar 100 µm.

We first investigated this effect in our patient cohort. Muscle biopsies were cut into sections and stained for type-1 and type-2 fibers by monoclonal antibodies for myosin light and heavy chain proteins. The staining with MHC slow and MHC fast antibodies were used to measure the size of the respective fiber types and the results were plotted for men and women (**Figure 3**).

Table 3. Summary of histological findings (mean values and standard deviation)

	Men			Women		
	Non sarcopenia (n = 5)	Probable sarcopenia (n = 7)	Sarcopenia (n = 2)	Non sarcopenia (n = 11)	Probable sarcopenia (n = 4)	Sarcopenia (n = 3)
Fiber type 1 [µm]	66.8 ± 17.7	55.1 ± 8.2	64.01 ± 14.1	57.9 ± 7.9	60.5 ± 9.0	68.6 ± 1.7
Fiber type 1 [%]	65.0 ± 16.0	59.7 ± 21.3	51.5 ± 0.7	58.8 ± 22.2	56.8± 20.0	61.6 ± 16.0
Mixed fiber type [%]	8.8 ± 7.5	9.9 ± 12.0	0.5 ± 0.7	15.1 ± 14.5	5.2 ± 4.6	2.9 ± 2.7
Fiber type 2 [µm]	59.1 ± 11.3	48.3 ± 3.9	34.5 ± 7.1	42.1 ± 7.6	42.9 ± 8.9	45.2 ± 6.5
Fiber type 2[%]	26.2 ± 14.8	30.4 ± 16.6	48.0 ± 1.4	26.1 ± 12.8	38.0 ± 16.1	35.5 ± 13.6
nMHC+ [%]	1.2 ± 1.3	3.9 ± 3.5	0.5 ± 0.7	1.3 ± 1.7	0.0 ± 0.0	1.3 ± 1.5
NCAM+ [%]	1.6 ± 2.4	0.9 ± 1.1	1.1 ± 1.5	2.9 ± 2.4	2.0 ± 3.4	3.0 ± 3.8
Pyknotic Nuclei [%]	1.5 ± 1.1	1.9 ± 1.1	0.8 ± 1.1	2.1 ± 2.7	3.2 ± 3.3	2.1 ± 1.5
PAX 7+ [%]	1.3 ± 0.8	1.1 ± 1.2	0.5 ± 0.7	0.6 ± 0.5	0.5 ± 0.9	0.7 ± 1.1

Table 4 shows the results of multiple linear regression analysis with Z-score sarcopenia as outcome **(figure 4)**.

• Model 1:

$$y = \beta_0 + \beta_1 x_1 + \varepsilon$$

• Model 2:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \varepsilon$$

• Model 3:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \varepsilon$$

Y= dependent variable; β_0 =y- intercept at time zero (costant term); β_1 is the coefficient of x_1 (the first characteristic histological data); β_2 is the coefficient of x_2 (sex); β_3 is the coefficient of x_3 (Age); x_1, x_2, x_3 indipendent variables; ε= the model error term.

Figure 4: Linear regression analysis equations used to calculate the following three models.

The beta value for histological data with Z-score as dependent variable (unadjusted model) shows significant value for atrophy factor type 2 fiber (β=0.520, p=0.002; model 1). But adjusted for sex there was a significant association for type 2 fiber diameter (β= -0.496, p=0.013), and atrophy factor type 2 fiber (β=0.485, p=0,008), (model 2). In the model adjusted for sex and age a significant association was found for type 2 fiber (β=433, p=0.030) and atrophy factor type 2 fiber (β=0.466, p=0,019) (model 3).

Table 4. Results of multiple linear regression analysis with Z-score of sarcopenia as dependent variable.

Independent variable	Model 1 β (*p*- value)	Model 2 β (*p*- value)	Model 3 β (*p*- value)
Fiber type 1 diameter	-0.049 (0.791)	-0.055 (0.760)	-0.028 (0.878)
Fiber type 2 diameter	-0.280 (0.120)	-0.496 (0.013)**	-0.433 (0.030)*
Hypertrophy factor fiber type 1	-0.097 (0.596)	- 0.042 (0.819)	-0.016 (0.933)
Hypertrophy factor fiber type 2	-0.233 (0.199)	-0.298 (0.096)	-0.287 (0.108)
Atrophy factor fiber type 1	0.197 (0.279)	0.065 (0.763)	0.070 (0.744)
Atrophy factor fiber type 2	0.520 (0.002)**	0.485 (0,008)**	0.466 (0.019)*
Fiber type 1 proportion	-0.008 (0.965)	-0.020 (0.911)	-0.052 (0.773)
Fiber type 2 proportion	0.223 (0.220)	0.213 (0.232)	0.242 (0.173)
Mixed fiber type proportion	-0.273 (0.131)	-0.243 (0.174)	-0.226 (0.204)
nMHC proportion	0.053 (0.772)	-0.035 (0.851)	0.051 (0.799)
NCAM proportion	-0.146 (0.425)	-0.086 (0.641)	0.039 (0.857)
Pyknotic nuclei proportion	-0.012 (0.946)	0.038 (0.836)	-0.012 (0.951)
Pax 7 proportion	-0.204 (0.263)	-0.314 (0.088)	-0.326 (0.073)

Model 1: unadjusted
Model 2: adjusted for sex
Model 3: adjusted for sex, age
Statistically significant result: *p-value ≤0.05, **p-value ≤0.01

Table 5 shows the results of multiple linear regression analysis with SMI as dependent variable, and for unadjusted model 1 the significant values found were type 2 fiber diameter (β=0.462, *p*=0.008) and Pax7 proportion (β=0.439, *p*=0.012). Model 2, adjusted for sex, showed significant values for type 2 fiber diameter (β= 0.353, *p*=0.048), atrophy factor type 2 fiber (β=-0.441, *p*=0.011) and Pax 7 (β=0.346, *p*=0.044). In model 3, adjusted for sex and age, significant associations were found in type 2 fiber diameter (β= 0.401, *p*=0.039), atrophy factor type 2 fiber (β=-0.509, *p*=0.007) and Pax 7 (β=0.348, *p*=0.047).

Table 5. Results of multiple linear regression analysis with SMI as dependent variable

Independent variable	Model 1 β (*p*- value)	Model 2 β (*p*- value)	Model 3 β (*p*- value)
Fiber type 1 diameter	0.041 (0.823)	0.032 (0.851)	0.031 (0.861)
Fiber type 2 diameter	0.462 (0.008)**	0.353 (0.048)*	0.401 (0.039)*
Hypertrophy factor fiber type 1	-0.056 (0.762)	-0.031 (0.858)	0.030 (0.867)
Hypertrophy factor fiber type 2	0.314 (0.080)	0.243 (0.155)	0.243 (0.163)
Atrophy factor fiber type 1	0.204 (0.263)	-0.035 (0.863)	-0.036 (0.864)
Atrophy factor fiber type 2	-0.223 (0.219)	-0.441 (0.011)**	-0.509 (0.007)**
Fiber type 1 proportion	0.071 (0.699)	0.053 (0.755)	0.056 (0.747)
Fiber type 2 proportion	-0.192 (0.291)	-0.209 (0.214)	-0.214 (0.215)
Mixed fiber type proportion	0.130 (0.478)	0.183 (0.281)	0.183 (0.291)
nMHC proportion	0.141 (0.441)	0.015 (0.935)	0.011 (0.957)
NCAM proportion	-0.190 (0.297)	-0.099 (0.572)	-0.148 (0.480)
Pyknotic nuclei proportion	-0.083 (0.653)	-0.009 (0.957)	-0.006 (0.972)
Pax 7 proportion	0.439 (0.012)**	0.346 (0.044)*	0.348 (0.047)*

Model 1: unadjusted
Model 2: adjusted for sex
Model 3: adjusted for sex, age
Statistically significant result: **p*-value ≤0.05, ***p*-value ≤0.01

In addition, multiple linear regression analysis with handgrip strength as dependent variable was calculated (**Table 6**). Significant results were found in unadjusted model 1, for type 2 fiber diameter (β= 0.574, *p*=0.001) and hypertrophy factor type 2 fiber (β=0.383, *p*=0.030).

Table 6. Results of multiple linear regression analysis with handgrip strength as dependent variable:

Independent variable	Model 1 β (*p*- value)	Model 2 β (*p*- value)	Model 3 β (p- value)
Fiber type 1 diameter	0.122 (0.540)	0.102 (0.539)	0.064 (0.693)
Fiber type 2 diameter	0.574 (0.001)**	0.469 (0.006)**	0.405 (0.023)*
Hypertrophy factor fiber type 1	0.000 (0.999)	0.097 (0.569)	0.060 (0.718)
Hypertrophy factor fiber type 2	0.383 (0.030)*	0.307 (0.063)	0.290 (0.068)
Atrophy factor fiber type 1	0.187 (0.307)	-0.089 (0.655)	-0.096 (0.615)
Atrophy factor fiber type 2	-0.208 (0.254)	-0.439 (0.010)**	-0.370 (0.040)*
Fiber type 1 proportion	0.012 (0.949)	-0.008 (0.962)	0.037 (0.820)
Fiber type 2 proportion	-0.112 (0.542)	-0.130 (0.435)	-0.168 (0.292)
Mixed fiber type proportion	0.124 (0.498)	0.182 (0.275)	0.157 (0.328)
nMHC proportion	0.165 (0.367)	0.028 (0.872)	-0.099 (0.583)
NCAM proportion	0.093 (0.614)	0.210 (0.216)	0.065 (0.736)
Pyknotic nuclei proportion	-0.104 (0.572)	-0.024 (0.887)	0.048 (0.774)
Pax 7 proportion	0.322 (0.073)	0.205 (0.235)	0.222 (0.179)

Model 1: unadjusted
Model 2: adjusted for sex
Model 3: adjusted for sex, age
Statistically significant result: *p-value ≤0.05, **p-value ≤0.01

Model 2, adjusted for sex, showed significant values for type 2 fiber diameter (β= 0.469, p=0.006) and atrophy factor type 2 fiber (β=-0.439, p=0,010). In model 3, adjusted for sex and age, significant association were found in type 2 fiber diameter (β= 0.405, p=0.023) and atrophy factor type 2 fiber (β=-0.370, p=0,040).

Multiple linear regression analysis with nMHC, NCAM, pyknotic nuclei and Pax 7, as independent variables were made also with Z-score, SMI and handgrip strength as dependents variables **(Tables 4, 5, 6).** No significant results were found, except for Pax 7. We found a significant association between Pax7 and SMI as dependent variables. For unadjusted model 1 the significant value found was β=0.439, *p*=0.012. Model 2, adjusted for sex, showed significant value Pax 7, β=0.346, *p*=0,044. In model 3, adjusted for sex and age, significant association was β=0.348, *p*=0,047 (**Table 5**).

We saw a decrease of type-1 fiber and type-2 fiber diameters at age above 70 for women and a decrease of type-2 fiber diameter in men (**Figure 5**). Looking at the different groups the samples with sarcopenia are below the regression line for men in fiber type-2 and above the regression line for women in fiber type-1 (**Figure 5**).

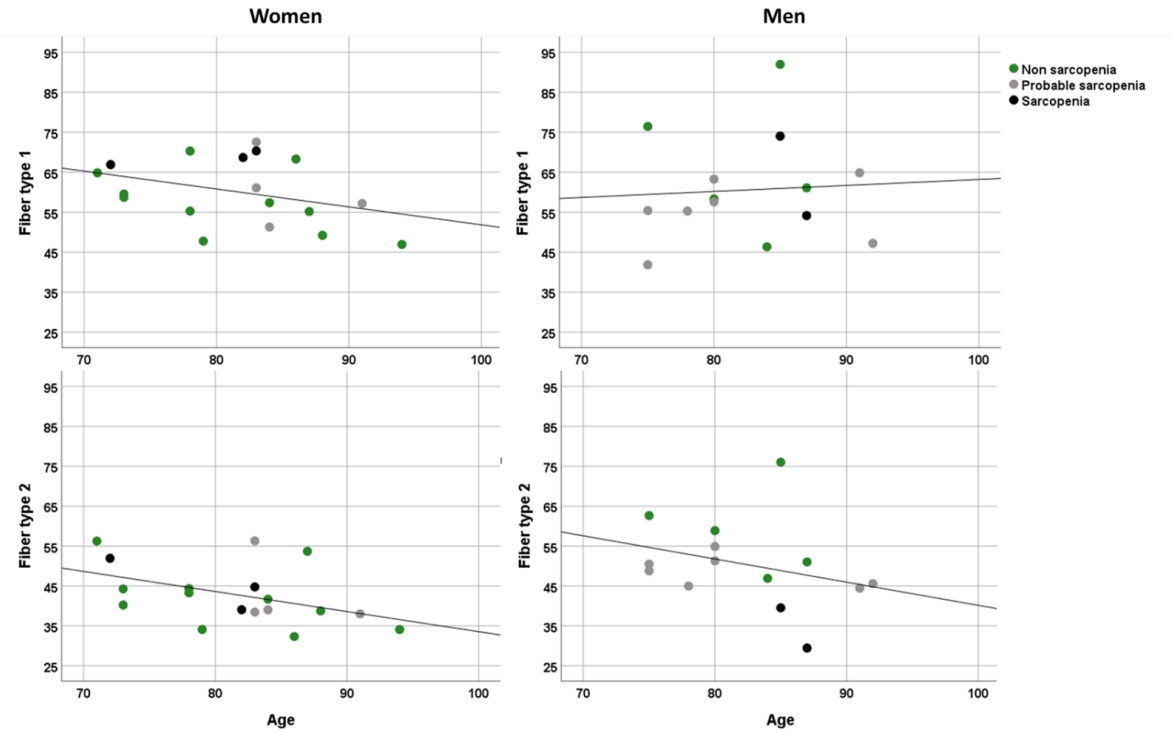

Figure 5. Mean fiber type-1 and -2 diameter in relation to age. The three groups are displayed in different colors and shapes.

Boxplots were used to illustrate the fiber size differences among the three groups in men and women. Statistical analysis revealed significant differences in fiber type-1 between non-sarcopenia and sarcopenia in women (p=0.037; **Figure 6**). For men we found significant differences among all three groups for fiber type-2 (ANOVA test, p=0.008; **Figure 6**).

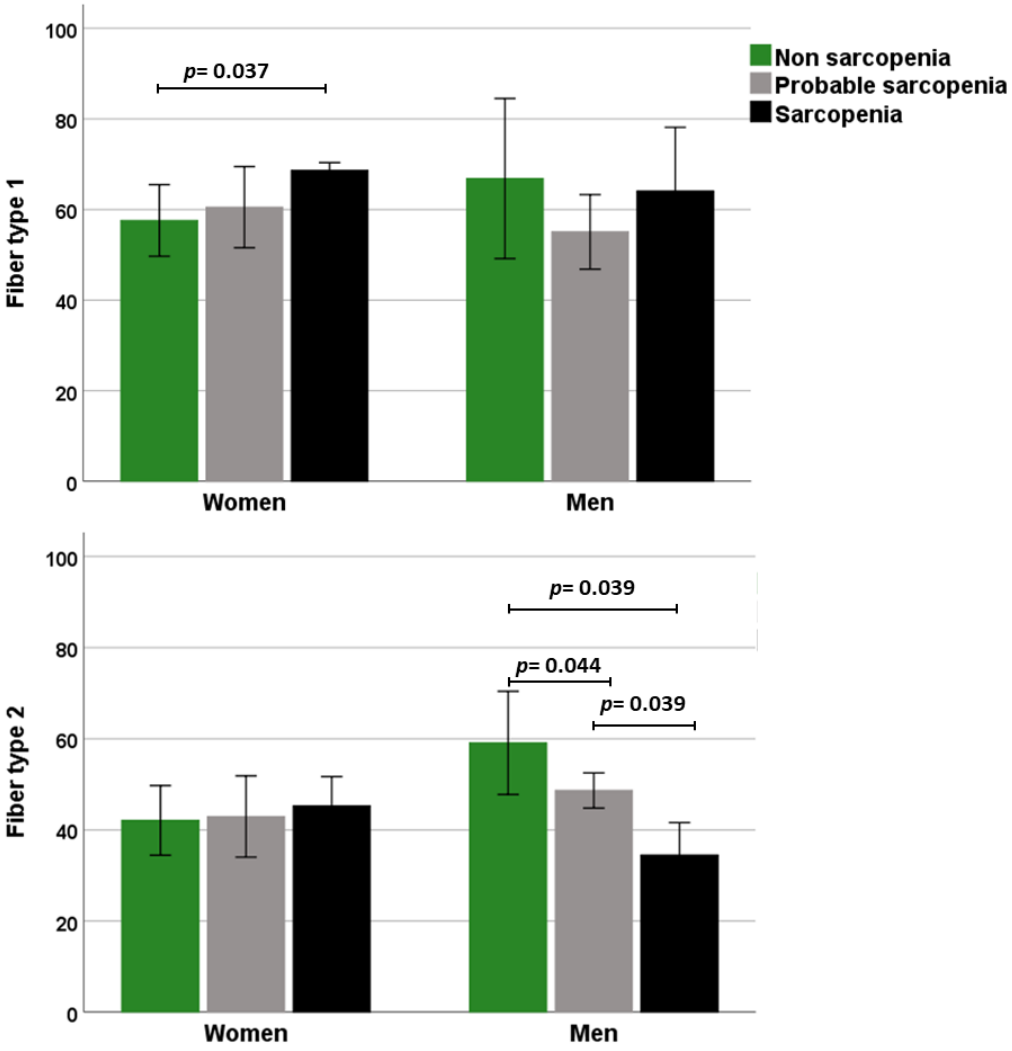

Figure 6. Fiber type-1 and -2 diameter compared between the different clinical classifications. *p* values between groups were calculated using two-sample-t-test.

In a next step we probed for the ratio of the different fiber types. For this muscle sections were co-stained for type-1 and type-2 fibers which were visualized using fluorescent secondary antibodies. Counting of type-1, type-2, and mixed fibers (positive for both antibodies) did show an increased proportion of type-2 fibers in sarcopenic men and in general less mixed fibers in sarcopenic men and women (**Figure 7**). The results of both stainings are summarized in **table 3**.

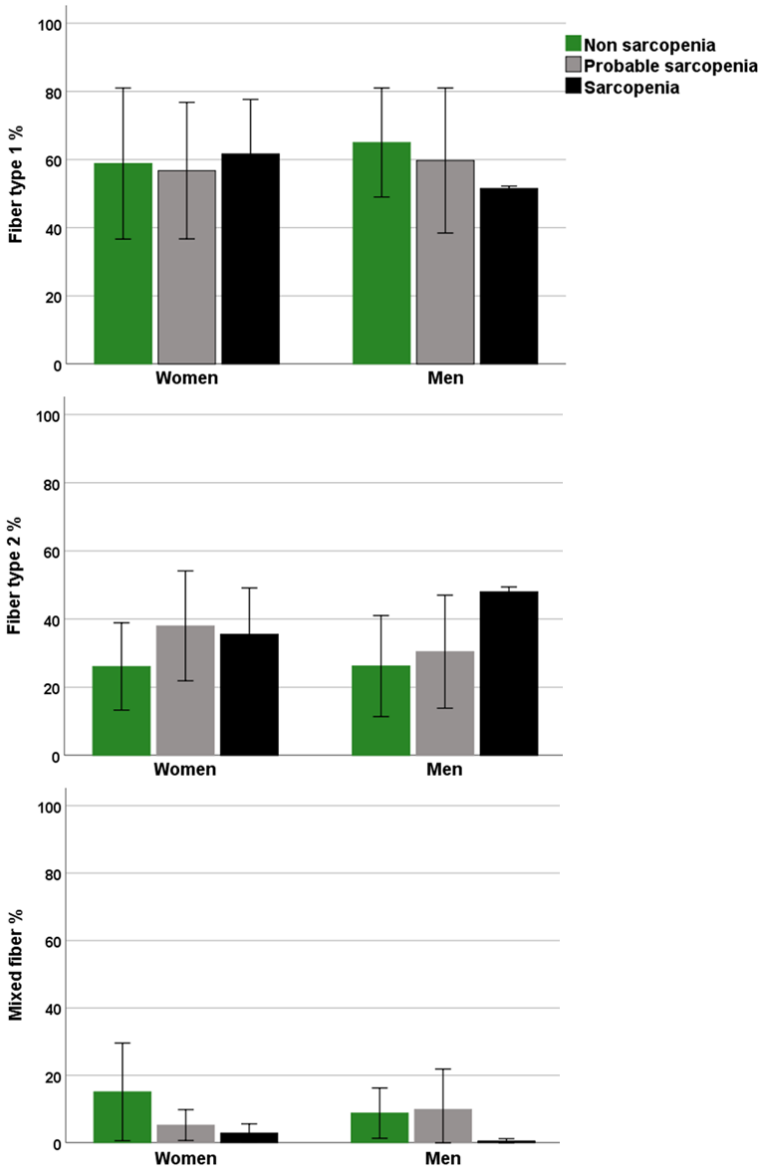

Figure 7. Percentage of fiber types-1, -2, and mixed fiber percentage compared between the different clinical classifications and both sexes.

3. Expression of denervation markers

Muscle denervation is an age-related phenomenon (Demontis et al., 2013; Rudolf et al., 2016). So, in order to study this on our population, our biopsies were cut and stained with specific antibodies for denervation NCAM (**figure 13**) and nMHC (**figure 15**). Pyknotic nuclei, another marker of denervation, were also analyzed with H&E staining (**figure 2**). In addition, an antibody capable of marking satellite cells, PAX7 (**figure 8**), was used to label muscle biopsies together with alfa dystroglycan.

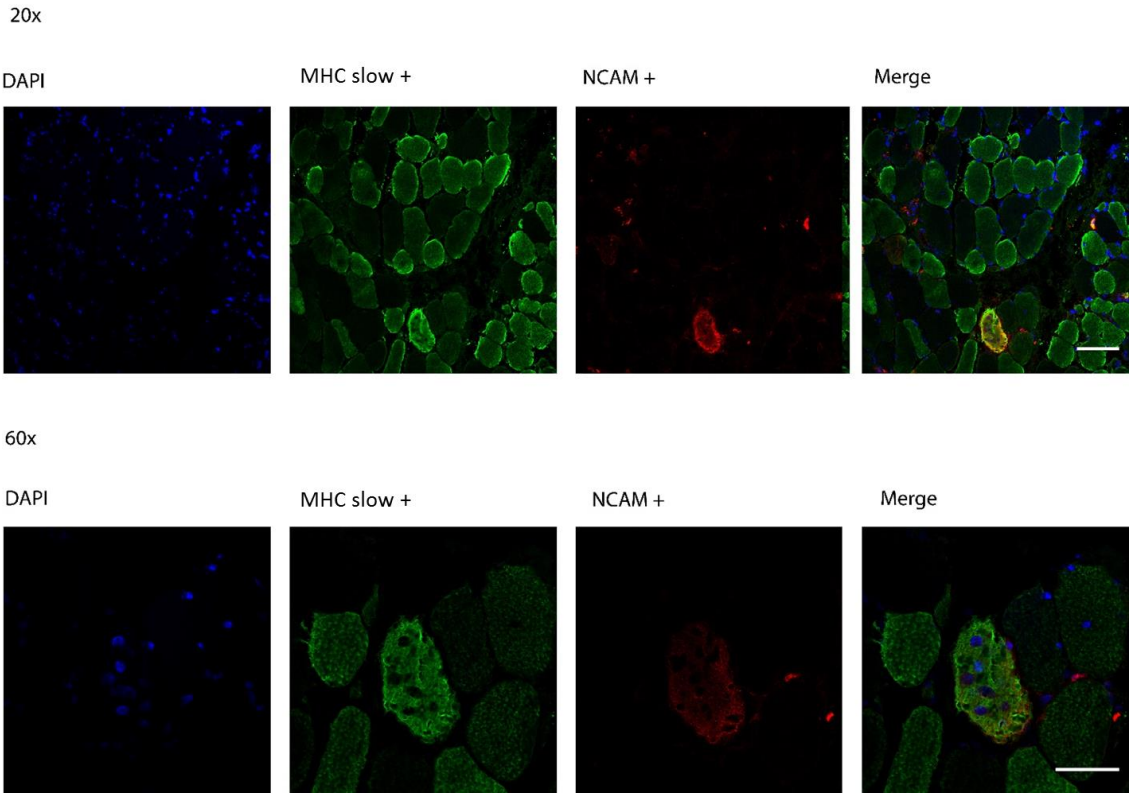

Figure 8. Co-staining with immunofluorescence nuclei in blue, MHCslow in green (fiber type 1) and NCAM+ in red (denervated fiber). Two different magnifications were used (20x and 60x). Scale bar 100 µm.

Boxplots were used to illustrate the differences between the three groups in men and women. No significant differences were found (**Figure 9**).

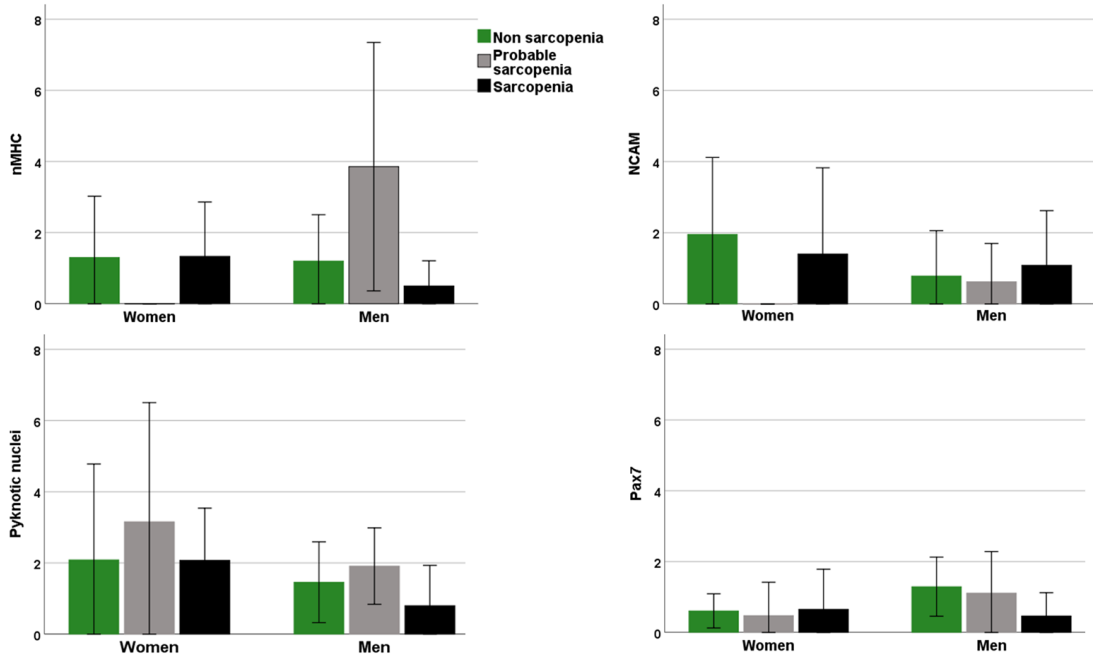

Figure 9. Percentage of nMHC, NCAM, Pyknotic nuclei and Pax7 percentage compared between the different clinical classifications and both sexes.

Multiple linear regression analysis was used, in addition, with MUNIX and MUSIX value as dependent variables, but no significant results were found (**table 7** and **8**).

Table 7. Results of multiple linear regression analysis with MUNIX as dependent variable

Independent variable	Model 1 β (p- value)	Model 2 β (p- value)	Model 3 β (p- value)
Fiber type 1 diameter	-0.145 (0.452)	-0.140 (0.472)	-0.188 (0.314)
Fiber type 2 diameter	-0.073 (0.705)	-0.021 (0.918)	-0.164 (0.435)
Hypertrophy factor fiber type 1	-0.072 (0.712)	-0.105 (0.597)	-0.148 (0.436)
Hypertrophy factor fiber type 2	-0.127 (0.511)	-0.098 (0.621)	-0.120 (0.530)
Atrophy factor fiber type 1	0.094 (0.629)	0.259 (0.259)	0.248 (0.259)
Atrophy factor fiber type 2	-0.015 (0.937)	0.062 (0.771)	0.237 (0.279)
Fiber type 1 proportion	-0.031 (0.875)	-0.031 (0.875)	0.028 (0.548)
Fiber type 2 proportion	-0.006 (0.974)	0.006 (0.974)	-0.047 (0.806)
Mixed fiber type proportion	0.056 (0.773)	0.041 (0.836)	0.013 (0.945)
nMHC proportion	0.217 (0.257)	0.300 (0.137)	0.191 (0.365)
NCAM proportion	0.041 (0.832)	0.006 (0.975)	-0.250 (0.262)
Pyknotic nuclei proportion	0.058 (0.765)	0.029 (0.885)	0.148 (0.455)
Pax 7 proportion	0.162 (0.400)	0.215 (0.277)	0.223 (0.241)

Model 1: unadjusted
Model 2: adjusted for sex
Model 3: adjusted for sex, age
Statistically significant result: *p-value ≤0.05, **p-value ≤0.01

Table 8. Results of multiple linear regression analysis with MUSIX as dependent variable

Independent variable	Model 1 β (p- value)	Model 2 β (p- value)	Model 3 β (p- value)
Fiber type 1 diameter	0.196 (0.308)	0.187 (0.321)	0.183 (0.344)
Fiber type 2 diameter	0.335 (0.076)	0.272 (0.169)	0.285 (0.183)
Hypertrophy factor fiber type 1	0.075 (0.698)	0.130 (0.498)	0.125 (0.525)
Hypertrophy factor fiber type 2	0.223 (0.245)	0.173 (0.368)	0.171 (0.385)
Atrophy factor fiber type 1	0.027 (0.890)	-0.179 (427)	-0.181 (0.431)
Atrophy factor fiber type 2	-0.125 (0.519)	-0.288 (0.158)	-0.310 (0.168)
Fiber type 1 proportion	-0.092 (0.633)	-0.93 (0.626)	-0.086 (0.662)
Fiber type 2 proportion	0.040 (0.838)	0.018 (0.924)	0.010 (0.959)
Mixed fiber type proportion	0.096 (0.619)	0.124 (0.513)	0.121 (0.534)
nMHC proportion	-0.148 (0.445)	-0.264 (0.181)	-0.333 (0.122)
NCAM proportion	0.186 (0.333)	0.258 (0.176)	0.319 (0.164)
Pyknotic nuclei proportion	-0.128 (0.507)	-0.079 (0.682)	-0.069 (0.740)
Pax 7 proportion	-0.217 (0.259)	-0.303 (0.113)	-0.302 (0.121)

Model 1: unadjusted
Model 2: adjusted for sex
Model 3: adjusted for sex, age
Statistically significant result: *p-value ≤0.05, **p-value ≤0.01

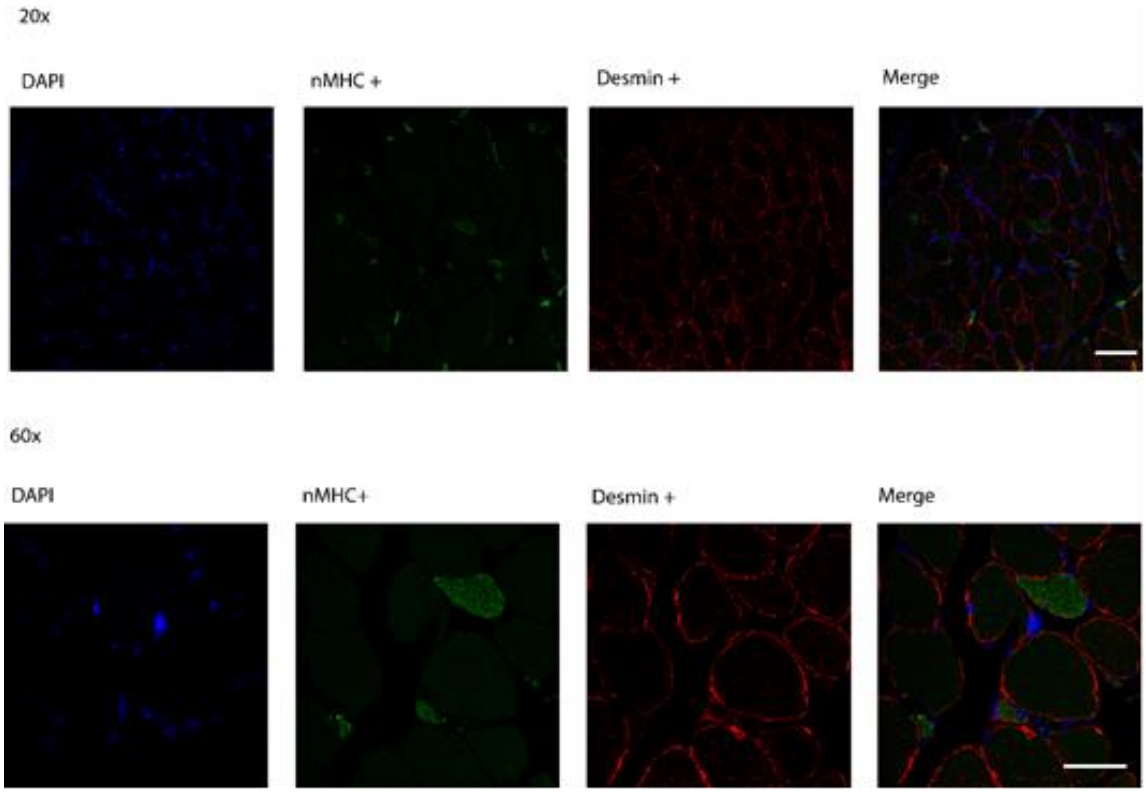

Figure 10. Co-staining with nuclei in blue, nMHC+ in green (denervated fiber) and Desmin+ in red (muscle fiber). Two different magnifications were used (20x and 60x) Scale bar 100 µm.

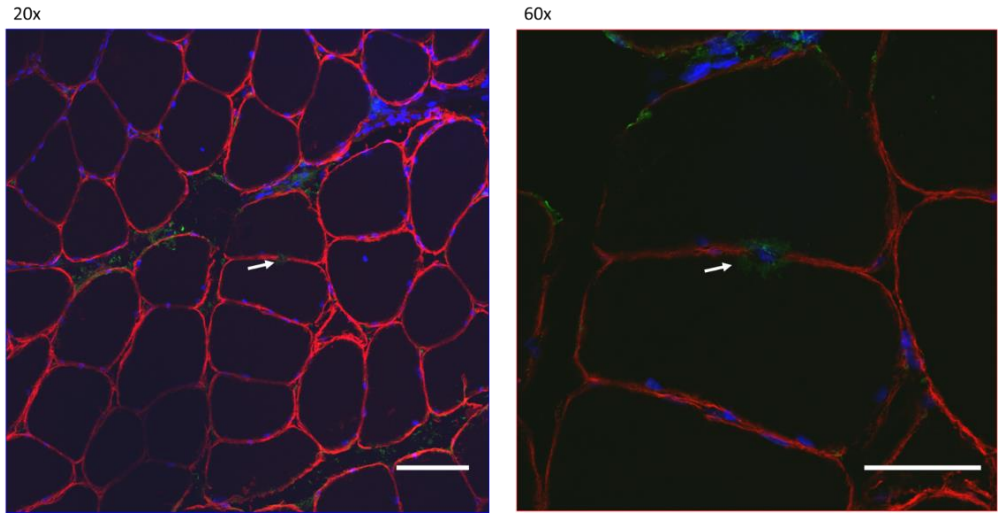

Figure 11. Satellite cells (arrow) express the transcription factor Pax7. Two different magnifications were used (20x and 60x). Scale bar 100 µm.

By dividing our population by sex a bivariate correlation has been made (**tables 9** and **10**). Considering the motor unit, an inverse correlation was found between MUNIX and PAX 7% (r = -0.527 *p*= 0.031) in women and a correlation with direct linearity between MUNIX and NCAM (r = 0.793 *p*= 0.002) in men.

Table 9. Correlation between neurogenic biomarkers and clinical data in women:

	nMHC r (p- value)	NCAM r (p- value)	Pyknotic nuclei r (p- value)	Pax 7 r (p-value)
Age	-0.195 (0.439)	**-0.522 (0.026)**	0.339 (0.168)	0.841 (0.871)
ASM	-0.350 (0.154)	-0.057 (0.822)	-0.084 (0.741)	0.341 (0.166)
Hand grip	0. 294(0.237)	0.310 (0.210)	0.140 (0.580)	0.013 (0.960)
Z-score	0.034 (0.894)	-0.152 (0.548)	0.134 (0.596)	-0.197 (0.433)
MUNIX	0.118 (0.651)	0.190 (0.465)	0.041 (0.866)	0.087 (0.749)
MUSIX	-0.115 (0.661)	-0.184 (0.479)	-0.109 (0.677)	**-0.523 (0.031)**

An inverse correlation between nMHC and age (r=-0.546 *p*=0.043) was found in men and a correlation between NCAM and age (r= -0.575 *p*=0.032; r= -0.522 *p*= 0.026) was found in men and women respectively (**tables 9** and **10**).

Table 10. Correlation between neurogenic biomarkers and clinical data in men:

	nMHC r (p- value)	NCAM r (p- value)	Pyknotic nuclei r (p- value)	Pax 7 r (p-value)
Age	**-0.546 (0.043)**	**-0.575 (0.032)**	0.116 (0.694)	0.068 (0.816)
ASM	0.243 (0.402)	-0.213 (0.465)	0.194 (0.507)	0.388 (0.171)
Hand grip	-0.096 (0.743)	0.158 (0.590)	0.205 (0.482)	0.365 (0.200)
Z-score	-0.080 (0.785)	0.030 (0.918)	-0.218 (0.455)	0.411 (0.144)
MUNIX	0.457 (0.135)	-0.454 (0.139)	0.301 (0.342)	0.363 (0.246)
MUSIX	0.393 (0.338)	**0.793 (0.002)**	-0.132 (0.683)	-0.240 (0.452)

G Discussions

The present study has shown a decrease in muscle fiber type-1 and fiber type-2 diameter age-related. Sarcopenia was characterized by a significant low fiber type-2 diameter in men. Percentages of mixed fibers, co-expressing both fiber types, were low in sarcopenic patients, indicating that denervation plays a minor role in the pathogenesis of sarcopenia in that cohort. But, at the same time, the denervation appears to be related to low number of motor unit and to age, especially in men.

1. Histomorphometric marker for sarcopenia

To our knowledge it is the first study investigating the histology of the vastus lateralis muscle in geriatric patients of both sexes (n=32, mean age=82.2 years, Table 1) in relation to their sarcopenia status (Table 2).

Two different staining protocols were used for this part of the project, to investigate on the one hand muscle fiber morphology (diameter, type differentiation**, Figure 3, 5)** and mixed fiber types on the other hand (marker of reinnervation, **Table 3, Figure 7**).

The decrease in the number and size of muscle fibers could result in the loss of skeletal muscle mass age dependent (Nilwik et al., 2013) with predominantly atrophy of type 2 fiber than fiber type 1 (Jan Lexell et al., 1986). According to these evidences, we found in our population an association between type 2 atrophy and a decrease in the muscle mass index (**table 5**).

Type-2 muscle fibers are involved in rapid muscle force production during muscle contraction, consequently, turn out to be important to find the posture to prevent a fall. Accordingly, the muscle strength of the quadriceps correlates positively with the size of the type 2 muscle fiber (Kramer et al., 2017). In

this regards a significant association was found between atrophy fiber type 2 and decrease of handgrip strength (**table 6**). The findings related to our study are reinforced by the association found between type 2 atrophy and degree of sarcopenia (**table 4**).

With regards for atrophy an age-related decrease in all samples and fiber types was found but an age-related increase was found in fiber type 2 diameter in men with sarcopenia, fiber type-1 in women with sarcopenia slightly above age-related decline. It is precisely in men with sarcopenia that there is a type 2 fiber atrophy, as the diameters of the fiber type-2 are mainly below the average range (**figure 6**, Dubowitz V, Sewry CA, Oldfors A Lane R, 2013). The results of table 3 indicate that higher ratio of fiber type-2 was found in sarcopenic men (**figure 7**). But, as previously reported, type 2 fiber hyperplasia found in this subpopulation is not in agreement with other studies (Nilwik et al. 2013).

Skeletal muscle fibers, known to have a great ability to adapt and improve elasticity, are the most abundant tissue cells in mammalian. Despite being completely differentiated and functionally integrated into the motor unit, muscle fibers under conditions of physiological and pathological needs can undergo a shift of the fiber type (Doran et al., 2007; Flück & Hoppeler, 2003).

As previously described in the introductory section, during the normal aging process, the loss of motor neurons occurs, especially those specific to type 2 muscle fibers. In these fibers, this process induces the coexpression of the two isoforms of myosin heavy chains (fast and slow) (Larsson et al., 1991). If denervation is more abundant than reinnervation, the fibers will undergo muscle atrophy and degeneration leading to sarcopenia (Jang & Van Remmen, 2011). In that cases mixed fibers can be a marker for sarcopenia based on denervation.

In our investigation the lowest percentage of mixed fibers was found in sarcopenic patients (1-3%, **Table 3**, **Figure 7**). This could lead to affirm that a denervation process leading to sarcopenia plays a minor role in such patients. But another possible conclusionit can be that the low percentage of mixed fibers in patients with sarcopenia may reflect a poor denervation capacity. This appears to be confirmed by the fact that the percentage of mixed fibers is higher in patients with probable sarcopenia (5-10%, **Table 3**) and even higher in non-sarcopenic patients (9-15%, **Table 3**).

Other studies have reported experiments in older adults with a percentage of mixed fibers above 28% (Andersen et al., 1999) but no information was provided on the sarcopenia status of that population. This leads us to assume that the participants were non-sarcopenic.

Currently, body activity and nutritional supplement are the only measure for the management of sarcopenia, therefore the present study in combination with further investigation can represent a profitable goal to better understand the genesis of skeletal muscle loss in order to prevent falls in the elderly population.

2. Denervation markers and sarcopenia

The multifactorial etiology of sarcopenia makes difficult discerning a temporal relationship between the changes at muscular level and the α-motoneuron level. During the aging motoneurons driving large, fast motor units (MU) seem susceptible to premature death, and these neurons die early due to unknown causes (Campbell et al., 1973; Jan Lexell, 1997; McNeil et al., 2005). Their death leaves seemingly healthy fast muscle fibers denervated, and the response of denervated fibers is normally to emit a “denervation signal” which initiates sprouting from nearby intact motor nerves or nerve termi-

nals. Sprouts grow to re-innervate denervated fibers with several structural and functional consequences.

First, surviving motor units tend to enlarge to incorporate a larger number of muscle fibers. Second, as surviving α-MNs are increasingly associated with “slow” muscle fibers (express MHCI)) there is an increasing tendency for re-innervated fibers to undergo transformation from fast (express MHCIIa, IIx/d or IIb) to slow types, and for slow fiber type clumping, (grouping of like fiber types together within the muscle) to occur. Several observations support the existence of each of these events (J. Lexell & Downham, 1991; Jan Lexell et al., 1986; Us, 2016). However, there is also evidence that some denervated fibers fail to become re-innervated by neighboring α-MNs, resulting in long-term denervation which has been shown to lead to muscle fiber atrophy (Rowan et al., 2012; Viguie et al., 1997). It has been proposed that an increase in MU size with decreasing numbers of neurons results in a declining ability to re-innervate (Siu & Gordon, 2003). If the capacity for neurons to reinnervate neighboring denervated muscles is retained with age, leads to the question of what alterations are causing the permanent denervation associated with atrophying fibers. One hypothesis is that absolute loss of α-MNs with age results in a decreased capacity for neuronal sprouting following denervation (Campbell et al., 1973; Rowan et al., 2012). The cause of α-MN death remains controversial despite many studies investigating age-related changes in neuron cell body number within the lumbosacral portion of the spinal cord. Tomlinson and Irving (1977) demonstrated that in humans there is no significant change in cell number until age 60, after which a negative correlation between cell number and patient age was observed. By age 90, a 70% decrease in α-MNs.

To calculate the number and size of motor neurons we used the MUNIX technique, as described in the method section. Considering the pathological value MUNIX below than 80 and MUSIX above than 100 (Drey et al., 2014) we can see in **table 2** how this match is present in men without sarcopenia. While, a pathological value of MUNIX (< 80) are shown in men with sarcopenia and women with probable sarcopenia.

The aim of this study was to test the hypothesis that denervation is related with sarcopenia.

Neurogenic marker, nMHC and NCAM, were used in order to study denervation in our population.

To justify the results of the following thesis, first of all, we should make a premise on the concept of satellite cell. Skeletal muscle satellite cells (SCs) are responsible for muscle growth during fetal and postnatal development (Buckingham, 2006). In adults, SCs are dormant myoblasts located between the plasma membrane and the basal lamina of muscle fibers, ready to differentiate for muscle regeneration when the muscle fiber is damaged (Lipton and Schultz 1979,MAURO, 1961)

One of the most common Abs used to study human SCs is anti-NCAM. Cells located at the periphery of myofibers showing a NCAM stained rim around a nucleus have been judged as SCs (Crameri et al., 2004; Verney et al., 2008).

During the fetal stage of human development nMHC is initially express in almost all muscle fibres initially (MHCn) (Barbet et al., 1991). nMHC a gradually replaced by adult MHC and upon birth only a minor proportion of muscle fibres express nMHC (Barbet et al., 1991).

Two studies have directly compared the prevalence of MHCn+ fibres in young and non-frail older individuals and found significantly higher levels in

the older (D'Antona et al., 2003; Mackey et al., 2014). Another marker which also has been used to identify SCs in human muscle is the paired-box transcription factor Pax7 (Crameri et al., 2007; Reimann et al., 2004), which is, unlike NCAM and nMHC, is proposed to be expressed only in SCs cells (Kuang & Rudnicki, 2008)

When a myofiber becomes denervated it also expresses Neural Cell Adhesion Molecule (NCAM) (Covault & Sanes, 1985; Messi et al., 2016), thought to be involved in the development of the neuro-muscular junction (Covault & Sanes, 1985; Walsh et al., 2000). The prevalence of NCAM + fibers in adult and unhealthy human muscles is believed to be practically nonexistent (Covault & Sanes, 1985).

In our population we found allow ratio for all these neurogenic markers. An inverse correlation between age and nMHC (r= -0.522 *p*=0.026) was found in men and a correlation between age and NCAM (r= -0.575 *p*=0.032; r= -0.522 *p*= 0.026) was notices in men and women respectively.

Being the loss of motor neurons linked to age, it could be that it is also connected with the increase in denervated fibers (Tomlinson & Irving, 1977). This could justify our results, therefore the re-expression of nMHC and NCAM in the elderly muscles. This suggest that with aging the denervation increases, in addiction no significant correlations have been found for the other neurogenic markers, as pyknotic nuclei.
As for Pax7, main marker for SCs (Reimann et al., 2004), low positive fibers were expected. But among our results an association was found between the SMI and Pax 7 (**table 5**) which leads to think how cell regeneration decreases with decreasing muscle mass.

Considering the whole investigation regarding the denervation, we can assume that the denervation appears to be related to the low number of motor

units and age, especially in men, but having found no correlation between MUNIX and Z-score, there is no relationship with sarcopenia in our cohort.

This last point can be explained due to the position of our measurements, since the MUNIX were calculated from the hand and the BIA from the leg.

3. Limitations

In interpreting the present findings, some limitations should be considered. Firstly, the study is descriptive in nature, observable by the small population (n=32). Secondly, being a cross-sectional study, causality in our results cannot be demonstrated. And to conclude, even though BIA is a technique used for the quantification of muscle mass, it is not considered a gold standard for estimating lean muscle mass like DXA.

H Conclusions

The results of this investigation show how fiber type 2 is atrophic in patients with sarcopenia, in particular in men. It has also been noticed that the regenerative capacity of the fiber is compromised in relation to muscle mass and that reinnervation does not occur in patients with sarcopenia.

It should also be noted that denervation is age-related but does not increase with sarcopenia in our study cohort.

The data obtained from this study are the first to be produced on the subject and therefore represent the basis for future and more complex investigations aiming to improving knowledge on the pathogenesis of sarcopenia.

I Contributions

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The clinical part and therefore the diagnosis of sarcopenia, including the measurement of MUNIX were conducted by medical students, Fabian Hofmeister, Stefanie Jarmusch, Lisa Baber. Dr. med Stefan Mehaffey and PD Dr. med Carl Neuerburg collected the vastus lateralis biopsy during surgery.

Part of these data has been reported in a paper under review by Tanganelli, Meinke et al., 2020.

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K Abbreviations

AF	Atrophy factor
aLM	Appendicular lean mass
BIA	Bioelectrical impedance analysis
DXA	Dual-energy X-ray absorptiometry
FT	Fiber type
HF	Hypertrophy factor
MHC	Myosin heavy chain
MN	Motor neuron
MU	Motor unit
Munix	Motor unit number index
Musix	Motor unit size index
NCAM	Neural Cell Adhesion Molecule
nMHC	Neonatal myosin heavy chain
Pax 7	Paired box 7
SMI	Skeletal muscle index
TBS	Tris-buffered saline

L List of the figures