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# Expression of sialic acids in human adult skeletal muscle tissue

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ABSTRACT

Investigations mostly in animal models have shown a role of sialic acid in the morphology and functionality of skeletal muscle during development and adult life. Several studies in humans have been performed regarding changes in sialic acid expression in a particular pathology, hereditary inclusion body myopathy, leading to muscular weakness and atrophy, with a similar phenomenon appearing also in sarcopenia of aging. In this study the expression of monomeric and polymeric sialic acids was evaluated in human skeletal muscle during adult life. Surgical biopsies of the Quadriceps femoris muscle from men aged 18–25 years (young group; $n = 8$) and men aged 72–78 (elderly group; $n = 10$) were collected for analysis. Expression of sialic acids was evaluated using lectin histochemistry, associated with enzymatic and chemical treatments to characterize monomeric and polymeric sialic acids. The polysialic acid expression was also evaluated by immunohistochemistry. Various types of sialic acid in the muscle tissue, in different amounts in the study groups, were detected. Monomeric sialic acids decreased in the elderly group compared with the young group, whereas polysialic acid increased. Sialic acid acetylation was present only in the young group. These findings demonstrated that changes in the expression of sialic acids in skeletal muscle tissue may be related to morphofunctional modifications occurring during aging.
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## Introduction

Sialic acids constitute a large family of nine carboxylated sugars. Because of their size, negative charge and position in glycoconjugate oligosaccharidic chains frequently terminal, they play an important role in maintaining membrane stability and modulation of various intercellular and/or intermolecular phenomena (Gabrielli et al., 2004; Accili et al., 2008; Schauer, 2009). Sialic acids possess structural diversities and different properties because of the addition of one or more O-acetyl esters to hydroxyl groups and the kind of linkage to the penultimate sugar (Angata and Varki, 2002; Schauer, 2004; Varki and Schauer, 2009). Linkage can be in configurations α2-3 or α2-6 with galactose or N-acetylgalactosamine, and in configuration α2-8 to form polysialic acids.

Investigations, mostly in animal models, have shown that sialic acid in skeletal muscle seems to play important roles in the functionality of various glycosylated molecules involved in fiber structure, excitability, regulation of contractile properties and neuromuscular junctions (Müthing et al., 1998; Hoyte et al., 2002; McDearmon et al., 2003; Johnson et al., 2004; Combs and Ervasti, 2005; Nilsson et al., 2010; Schwetz et al., 2011). During embryonic and post-natal development of skeletal muscle, changes in expression of this anionic molecule seem to be related to its differentiation and the onset of its functionality (Clark and Smith, 1983; Rafuse and Landmesser, 1996; Ziak et al., 1996).

Only limited data in animal models are available regarding the changes in the expression of sialic acid in skeletal muscle during aging, when loss of muscular strength and mass, a phenomenon called sarcopenia, occurs (Mitchell et al., 2012; Walston, 2012; Montero-Fernandez and Serra-Rexach, 2013). A study demonstrated sialic acid changes in a glycolytic muscle key enzyme leading to a decrease of its activity that in turn alters the

*Abbreviations:* ANOVA, analysis of variance; BSA, bovine serum albumin; DAB, diaminobenzidine; DBA, *Dolichos biflorus* agglutinin; DIG, digoxigenin; FBS, fetal bovine serum; GNE, UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase; HIBM, hereditary inclusion body myopathy; HRP, horseradish peroxidase; MAA, *Maackia amurensis* agglutinin; NCAM, neural cell adhesion molecule; NBT, nitroblue tetrazolium; OD, optical density; PBS, phosphate buffered saline; PNA, peanut agglutinin; PO, periodate oxidation; PSA, polysialic acid; SBA, soybean agglutinin; SNA, *Sambucus nigra* agglutinin; TBS, Tris buffered saline.

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metabolism of muscle fibers (O'Connell et al., 2008). Other studies showed that in muscle during adult life the degree of sialylation of the glycoprotein neural cell adhesion molecule (NCAM) seems to influence the re-innervation process, after experimental denervation (Olsen et al., 1995; Rønn et al., 2000).

Several studies performed in animal models and also in humans, have investigated the role of sialic acid in a particular pathological condition, hereditary inclusion body myopathy (HIBM), leading to muscular weakness and atrophy and which presents similar phenomena to sarcopenia. HIBM develops due to mutations of the UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE) gene involved in sialic acid biosynthesis (Huizing and Krasnewich, 2009). Data have demonstrated that hyposialylation of glycosylated molecules needed for the correct structure and functionality of muscle tissue, such as NCAM, α-distroglycan and neprilysin, seem to alter their activity (Saito et al., 2004; Tajima et al., 2005; Broccolini et al., 2008, 2009, 2010, 2011; Jay et al., 2008; Huizing and Krasnewich, 2009; Jay et al., 2009; Nilsson et al., 2010; Voermans et al., 2010; Valles-Ayoub et al., 2012). In some investigations it has been suggested to use sialic acid and/or its metabolites as a prophylactic treatment for HIBM (Huizing and Krasnewich, 2009; Malicdan et al., 2009, 2010, 2012; Niethamer et al., 2012; Nishino and Noguchi, 2012a, 2012b).

The aim of this study was to perform a broad characterization of monomeric and polymeric sialic acids and to evaluate their expression in human skeletal muscle tissue during adult life. This study was undertaken to determine possible age-related changes in the content and distribution of these molecules and to highlight their role in the structure and functionality of muscle. For this purpose lectin histochemistry and immunohistochemical methods were employed.

## Materials and methods

### Samples

Biopsy specimens of the right or left quadriceps muscle from men aged 18–25 years ($n = 8$, young group) and from men aged 72–78 ($n = 10$, elderly group) were obtained during orthopedic surgery early after trauma. The history of patients revealed the absence of skeletal muscle disease and only moderate physical activity. Samples were obtained with written consent from each subject, in accordance with the recommendations of the Ethical Committee on human experimentation (Marini et al., 2012). Muscle samples were fixed in 10% neutral buffered formalin solution for 12 h and routinely processed and embedded in paraffin wax and cut as 5 μm-thick sections.

### Histology

Some sections were stained with hematoxylin-eosin (H&E) for histological investigation by light microscopy (Microphot-FXA, Nikon, Tokyo, Japan). The diameter of muscle fibers and the connective area among muscle fascicles were evaluated by scanning 10 random optical fields (600,625 $\mu m^2$; 40× objective), using ImageJ software (NIH, Bethesda, MD, USA) in each section (5 for each specimen). The measurements were examined by two different observers unaware of the source of the slides ("blind study").

### Sialic acid expression analysis

### Lectin histochemistry

Two methods for lectin histochemistry were used: the 'direct' and the 'indirect' technique. In the 'direct' technique, *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA) were used to identify sialic acids linked α2-3 and α2-6 to galactose or galactosamine, respectively (Shibuya et al., 1987; Wang et al., 1988; Gabrielli et al., 2004; Adembri et al., 2011; Marini et al., 2011; Adembri et al., 2014). In the second method peanut agglutinin (*Arachis hypogaea*) (PNA), soybean agglutinin (*Glycine maximus*) (SBA) and *Dolichos biflorus* agglutinin (DBA) combined with neuraminidase digestion, deacetylation and differential oxidation to reveal acetylic groups, were used to investigate the expression of sialic acid linked to D-Gal(β1→3)-D-GalNAc, to α/βD-GalNAc > D-Gal and αD-GalNAc respectively, and the structure of sialic acids (Gabrielli et al., 2004; Adembri et al., 2011; Marini et al., 2011; Adembri et al., 2014).

Table 1 summarizes the lectins and their specific sugar residues.

MAA, SNA and PNA were digoxigenin (DIG) labeled lectins (Roche Diagnostics, Mannheim, Germany). SBA and DBA were horseradish peroxidase (HRP) conjugated lectins (Sigma–Aldrich, St. Louis, MO, USA).

**DIG-labeled lectins.** After hydration, sections were treated with 20% acetic acid for 15 min at 4 °C, to inhibit the endogenous alkaline phosphatase, rinsed in distilled water and treated with 10% blocking reagent (Roche Diagnostics, Mannheim, Germany) in TBS (Tris–HCl 0.05 M, NaCl 0.15 M, pH 7.5) for 30 min to reduce the background labeling. Then sections were washed twice in TBS for 10 min and rinsed in Buffer 1 ($MgCl_2$ 1 mM, $MnCl_2$ 1 mM, $CaCl_2$ 1 mM in TBS, pH 7.5) for 10 min. Sections were then incubated in DIG-labeled lectins diluted in Buffer 1 for 1 h at room temperature. Lectins were solutions (1 mg/1 mL) in 50 mM Tris–HCl, 0.05% sodium azide. The optimal dilution for each lectin, which allowed the maximum labeling with minimum background, was 1 μL/mL, 5 μL/mL and 10 μL/mL for SNA, MAA and PNA, respectively. Sections were then rinsed three times for 10 min in TBS, incubated with anti-digoxigenin (0.3 mL polyclonal sheep anti-digoxigenin Fab fragments), conjugated with alkaline phosphatase (750 U/mL) (Roche Diagnostics, Mannheim, Germany) diluted in TBS (1 μL/1 mL) for 1 h and washed three times in TBS for 10 min. Labeling of the sites containing bound lectin-digoxigenin was obtained by incubating slides with Buffer 2 (Tris–HCl 0.1 M, $MgCl_2$ 0.05 M, NaCl 0.1 M, pH 9.5), containing nitroblue tetrazolium (NBT)/X-phosphate (20 μL/1 mL), for 10 min at room temperature.

**HRP-conjugated lectins.** After hydration, sections were treated with 3% hydrogen peroxide for 10 min to inhibit the endogenous peroxidase, rinsed in distilled water and treated with 0.1% bovine serum albumin (BSA) in TBS (0.05 M Tris–HCl, 0.15 M NaCl, pH 7.5) for 20 min, to reduce the background labeling. Sections were then incubated for 30 min at room temperature in HRP-conjugated lectins solution in TBS. The optimal concentration for lectin, which allowed the maximum labeling with minimum background, was 20 μL/mL and 25 μL/mL for SBA and DBA, respectively. Then sections were rinsed three times in TBS. Visualization of the sites containing bound lectin-HRP was achieved by incubating slides in 3,3′ diaminobenzidine tetrahydrocloride (DAB) (Sigma Fast DAB tablet set, Sigma–Aldrich, St. Louis, MO, USA) for 10 min at room temperature.

All specimens were rinsed in distilled water, dehydrated using graded ethanol solutions, cleared in xylene and mounted in Permount™ (Fisher Scientific, Fair Lawn, NJ, USA).

**Enzymatic and chemical treatments.** In some experiments, sialic acid was removed by pretreating sections for 18 h at 37 °C in a solution of sodium acetate buffer 0.25 M, pH 5.5, containing 0.1 unit/ml sialidase (Type X from *Clostridium perfringens* specific for sialic acids linked α2-3, α2-6 and α-2,8; Sigma–Aldrich, St. Louis, MO, USA), 50 mM $CaCl_2$ and 154 mM NaCl, before staining with PNA, SBA and DBA. Deacetylation was performed by incubating sections with 0.5% KOH in 70% ethanol for 30 min at room temperature. This

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