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Observations of nemaline bodies in muscle biopsies of critically ill patients infected with SARS-CoV-2

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Mini abstract

This study describes the presence and morphology of typical nemaline bodies in skeletal muscle tissue from critically ill patients infected with SARS-CoV-2, which should be interpreted primarily as a non-specific pathological response of extreme myofibrillar disintegration associated with myofiber necrosis.

Figure 4

Abstract

Patients infected with SARS-CoV-2 who have been admitted to the intensive care unit (ICU) often face months of physical disability after discharge. To optimize recovery, it is important to understand the role of musculoskeletal alterations in critically ill patients infected with SARS-CoV-2.

The main aim of the present study was to describe the presence and morphology of nemaline bodies found in skeletal muscle tissue from critically ill patients infected with SARS-CoV-2.

In *n*=7 patients infected with SARS-CoV-2, ultrastructural characteristics of *vastus lateralis* muscle obtained on days 1-3 (T0) and days 5-8 (T1) following ICU admission were investigated in more detail with electron microscopy. Those muscle biopsies consistently showed variable degrees of myofiber necrosis and myofibrillar disorganisation. In 4/7 (57%) patients at T1, the Z-line material accumulated into nemaline bodies with a typical lattice-like appearance at higher magnification, similar to that found in nemaline myopathy.

This study is the first to describe the disintegration of myofibrils and accumulation of Z-line material into nemaline bodies in skeletal muscle tissue obtained from critically ill COVID-19 patients following ICU admission, which should be interpreted primarily as a non-specific pathological response of extreme myofibrillar disintegration associated with myofiber necrosis.

Introduction

Acute coronavirus disease (COVID)-19, is a serious vascular disease caused by the Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2) (1, 2) and has a heterogenous clinical presentation with primary respiratory symptoms (3, 4). The majority of patients infected with SARS-CoV-2 clinically presents with mild to moderate respiratory symptoms and will recover without requirement of special treatment (5). However, in older and in patients with comorbidities, SARS-CoV-2 infections often develop to critical illness with severe acute respiratory failure and accompanying multi-organ failure, requiring intensive care unit (ICU) admission and ventilatory support (6, 7).

Additionally, patients with SARS-CoV-2 infections often exhibit skeletal muscle-related symptoms, such as muscle pain (myalgia), muscle fatigue and muscle weakness which limit exercise capacity, impede functional recovery, compromise rehabilitation, and lower quality of life (8-12). The high prevalence of skeletal muscle-related symptoms suggests that structural skeletal muscle alterations are common in patients with COVID-19 (12). However, despite the clinical evidence, the impact of COVID-19 on skeletal muscle tissue remains mainly unknown. Until now, literature on the impact of SARS-CoV-2 infections on skeletal muscle tissue in critically ill patients is mostly limited to postmortem studies (13) and case reports (14-21).

Recently, we analyzed *vastus lateralis* biopsies from critically ill patients infected with SARS-CoV-2 on admission and after 7 days of ICU stay, and showed ultrastructural evidence for myofibrillar degeneration accompanied by hydropic degeneration and necrosis of myofibers (22). These ultrastructural alterations in muscles from critically ill patients infected with SARS-CoV-2 included Z-line abnormalities, myofilament loss and disintegration of myofibrils.

An extreme degree of sarcomeric disintegration is the accumulation of Z-line material into nemaline bodies or rods (23). The main aim of the present study was to describe the presence and morphology of nemaline bodies found in skeletal muscle tissue from critically ill patients infected with SARS-CoV-2.

Materials and Methods

This study is part of a monocentre, longitudinal, prospective observational cohort study which was approved by the Medical Ethical Committee of Hasselt University and of Jessa Hospital, registered at clinicaltrials.gov (NCT04698798) and conducted in accordance with the principles of the Helsinki Declaration 2013. All patients were included in the study after obtaining informed consent from the patient or legal representative. Patients diagnosed with COVID-19 pneumonia and admitted to the ICU of Jessa Hospital (Hasselt Belgium) were included in the study. A diagnosis of COVID-19 was confirmed according to the World Health Organization (WHO) protocol as a positive result on polymerase chain reaction (PCR) assays of nasopharyngeal swab samples or on bronchoalveolar lavage. Patients had to be 18 years of age or older, and had an expected ICU stay of more than seven days to be included in the study. At baseline, clinical characteristics, demographic data of the patients and severity of disease were recorded. These data included Acute Physiology and Chronic Health Evaluation II (APACHE II) score (24) and Sequential Organ Failure Assessment (SOFA) score (25). Muscle biopsies of the *vastus lateralis* were taken at two time points, at day 1-3 post admission (T0) and day 5-8 post first biopsy (T1), using a 12G semi-automatic Bard® Mission® Core Biopsy needle. Standard medical care was not changed during the study period. The muscle biopsy samples were cut into blocks of 1mm and immediately fixed by immersion in a solution of 2% glutaraldehyde in 0.05 M

sodium cacodylate buffer (pH 7.3) at 4° C and processed for electron microscopy. These specimens were post-fixed with 2% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.3) for 1 hour at room temperature, dehydrated in graded acetone, impregnated overnight in a 1:1 mixture of acetone and araldite epoxy resin at room temperature, followed by impregnation in 100% Araldite epoxy resin at 40°C for 3 h and finally embedded in Araldite epoxy resin at 60°C for 24 h. Semithin sections (0.5 µm) were cut with an ultramicrotome (Leica EM UC6 microtome) and stained with a solution of thionin and methylene blue (0.1 % aqueous solution) for light microscopic examination to identify nemaline bodies. Representative pictures were scanned with an Axioscan Z1 (Carl Zeiss Microscopy GmbH, Jena, Germany) and analyzed with Zen 3.3 (Blue edition) software (Carl Zeiss Microscopy GmbH, Jena, Germany).Ultrathin sections (70 nm) of selected tissue blocks were mounted on 0.7 % Formvar-coated copper grids (Aurion, Wageningen, the Netherlands), contrasted with 0,5% uranyl acetate and a stabilized solution of 3% lead citrate using a Leica EM AC20 (Leica Microsystems, Diegem, Belgium) and examined in a transmission electron microscope EM 208 (Philips, Eindhoven, The Netherlands) operated at 80 kV. Representative digital images were acquired by a Morada Soft Imaging System camera with associated ITEM-FEI software (Olympus SIS, Münster, Germany).

Results

The ultrastructural characteristics of the skeletal muscle fibers were investigated with electron microscopy performed on muscle tissue collected from 7 patients at T0 and T1. Patients' characteristics are displayed in Table 1.

INSERT TABLE 1

On electron microscopic examination, degenerative muscle fibers showed varying degrees of myofibrillar disintegration, presenting as focal areas of Z line and myofibrillar disorganization, both at T0 (figure 1) and T1 (figure 2). Among fibers with normal sarcomeric organization (figure 1A), fibers with identifiable sarcomeres but lost normal striation pattern (figure 1B) and others with focal Z line irregularities, smearing of the Z line material and myofilament disruption (figure 1C) were found at T0. Most degenerative myofibers at T1 showed ultrastructural evidence of extensive myofiber necrosis, diffuse myofibrillar degeneration and hydropic degeneration in individual fibers (figure 2). Noteworthy, myofibers presenting severe necrosis contained typical nemaline bodies (figure 2, insert C), showing the same electron density as the Z-line material of the sarcomeres. These nemaline bodies were found in biopsy samples taken at T1 in 4/7 (57%) patients.

INSERT FIGURE 1 AND 2

Thionin and methylene blue-stained sections showed prominent thread-like (figure 3A) and rod-shaped (figure 3B) particles in the myofibers with variation in number, size and distribution. Electron microscopy revealed that these particles appeared as electron-dense structures, that were identified as nemaline bodies (figure 4)..

INSERT FIGURE 3

Nemaline bodies were usually clustered, often in the perinuclear region or beneath the sarcolemma (figure 4-7). Their number and size varied extremely. Nemaline bodies can be parallel oriented and mostly thread-like (figure 4A) or chaotically oriented and more rod-like (figure 4B). The central part of nemaline bodies showed one or several cracks of low electron density.

INSERT FIGURE 4

In several myofibers, a clear transition between myofibrillar disintegration and nemaline body formation could be observed. In these myofibers, nemaline bodies were observed in the most necrotic part of the myofiber (figure 5). Necrotic myofibers containing nemaline bodies showed clustered nuclei and local hypercontraction of myofibrils.

INSERT FIGURE 5

Individual nemaline bodies were intimately related to the remaining myofilaments (figure 6). The shorter, thinner (actin) filaments are interposed between the longer, thicker (myosin) filaments, which they only partially overlap. Only the thin (actin) filaments are in continuity with the Z-line material of the nemaline bodies and seem to be anchored on their circumference, similar to the ultrastructural organization of the A- and I-band. Some the filaments might also be intermediate desmin filaments that connects the sarcomeres at the Z-line with mitochondria, the nucleus, the sarcolemma and the T-tubules(26).

INSERT FIGURE 6

At higher magnification nemaline bodies displayed a periodic pattern of lines parallel and perpendicular to the long axis of the nemaline bodies, which result in a lattice-like structure (figure 7). The electron density and lattice-like pattern of nemaline bodies are in continuity with the thin filaments.

INSERT FIGURE 7

Discussion

To our knowledge, this is the first ultrastructural study describing nemaline bodies in skeletal muscle tissue samples of COVID-19 patients following ICU hospitalization. The presence of nemaline bodies in skeletal muscle fibers is regarded as the pathological hallmark of nemaline myopathy (NM) (27, 28). Clinically, NM can be classified into a heterogeneous group of early, genetic (hereditary) forms, collectively termed congenital nemaline myopathies (29, 30), which have a chronic progressive course, and can develop into a rare adult form, with a subacute progression. The adult-onset forms are called adult-onset NM (31) or sporadic late-onset NM (SLONM) (32-36).

It is generally accepted that the primary defect in NM is myofibrillar damage in the sarcomeric thin filament resulting in the disintegration of the myofibrils into nemaline bodies. Our ultrastructural observations showed the formation of nemaline bodies in continuity with Z-line material, which is in line with the general belief that nemaline bodies are derived from Z-lines (37-39). The pathophysiological mechanism leading to nemaline body formation is poorly understood. However, it is believed that nemaline body formation is secondary to contractile dysfunction (40). A recent electron microscopic study in SLONM patients showed that a defective non-sarcomeric cortical cytoskeletal network (connecting the myonuclei) and decreased contractile force production cause abnormal morphology and positioning (e.g. clustering) of the myonuclei. These nuclear defects are assumed to compromise gene expression and myofiber integrity, contributing to the disintegration of sarcomeres (41). In the present study, we also observed clustered nuclei in the muscle fibers that showed severe contractile filament disarray and associated nemaline bodies, which is in line with the nuclear aberrations described in SLONM (41).

While the presence of nemaline bodies is a mandatory finding for the diagnosis of NM, finding nemaline bodies in a muscle biopsy sample is not pathognomonic for NM (42). Nemaline bodies can be found in myopathies related to various disorders (27, 43-45). Nemaline bodies can be found in HIV infection, which is referred to as HIV-associated NM (HIV-NM) (46-51). The myopathy seen in patients infected with HIV is considered an immune-mediated myopathy, in which viral particles were not found in the skeletal muscle tissue (52). It is still unclear whether HIV-NM has to be regarded as HIV-associated SLONM (51, 53, 54) or that HIV-NM and SLONM are different disease entities (34). Schnitzler et al. published a systematic review of the clinicopathological features in a large cohort of patients with SLONM compared to HIV-NM cases (34). Histopathologically, muscle biopsies in SLONM and HIV-NM showed the same electron microscopic alterations, including nemaline bodies, fiber atrophy, myofibrillar disintegration, mitochondrial and vacuolar changes, lobulated fibers and central nuclei. Compared to SLONM, the nemaline bodies of HIV-NM patients did not appear in the nucleus and are associated with a significantly higher degree of myofiber necrosis and inflammation (34, 47). The ultrastructural characteristics of the muscle fibers of the COVID-19 muscle tissue samples were very similar to those in SLONM and HIV-NM (34). Furthermore, there is an exceptional resemblance to the morphology of the lobulated fibers described by Chahin *et al.* (49) in SLONM, in which clustered nemaline-bodies are located centrally and/or garlanded by mitochondria. The lattice-like morphology of the nemaline bodies in COVID-19 muscle samples, which show crack-like structures of low electron density, as well as the orientation of actin and myosin filaments, is very similar to that found in a patient with polymyositis (27). Muscle samples from COVID-19 patients showed the presence of nemaline bodies along with the changes described for the necrotic myofibers in HIV-NM (34). Schnitzler *et al.* (34)

assumed that there are three possible pathophysiological explanations for the Z-line disorganization and nemaline body formation in HIV-NM: a direct effect of the viral particles, an immune-mediated process triggered by the virus or an HIV-caused genome alteration.

SARS-CoV-2 enters host cells by binding to the angiotensin converting enzyme 2 (ACE2) receptor. Skeletal muscle tissue only expresses the ACE2 receptor on satellite cells, fibroblasts and endothelial cells. To our knowledge, there are no conclusive reports on the presence of virus particles in skeletal muscle tissue. The absence of ACE2 receptors on skeletal muscle cells and the lack of evidence for the presence of viral particles within the myofiber suggest that a direct effect of the virus is rather unlikely. Infection with SARS-CoV-2 is known to increase levels of circulating pro-inflammatory cytokines and to induce systemic inflammation (55). This systemic inflammation can cause sepsis and ARDS, indicating an overactive immune response which can aggrevate skeletal muscle damage(21, 55). Logically, this implies that SARS-CoV-2-associated myopathy is rather a (post)infectious necrotizing process (21), consistent with the HIV-associated immune-mediated necrotizing myopathy, in which viral particles are also undetectable (52).

An important prerequisite for the diagnosis of SLONM is the presence of muscle symptoms in combination with large numbers of nemaline bodies (56). Furthermore, SLONM is not characterized by myonecrosis and typical features of muscle repair (i.e. regeneration with central-nucleated fibers) (54, 57, 58). In contrast to these observations, we found nemaline bodies in small quantities and a high degree of myofiber necrosis as well as regenerating central-nucleated muscle fibers (22). Therefore, when nemaline bodies were found in COVID patients, the myopathy associated with SARS-CoV-2 can be classified as a myopathy with nemaline bodies,

rather than a nemaline myopathy, since the nemaline bodies occur non-specifically due to myofibrillar breakdown (59).

Significance, perspectives and limitations

In this study, we demonstrated the presence of nemaline bodies in skeletal muscle tissue obtained from critically ill patients infected with SARS-CoV-2 following ICU admission. As a control group of ICU patients without SARS-CoV-2 infection is not included in this study, we cannot determine whether nemaline bodies are also present in non-COVID-19 ICU patients. To our knowledge, the presence of nemaline bodies has not been described in muscle tissue obtained from critically ill patients. Preclinical data from a mouse model of NM showed that immobilization resulted in an increase in the number of nemaline bodies accompanied by myofiber atrophy and severe muscle weakness. The disuse-induced muscle weakness was proportionally associated with the number of nemaline bodies. Endurance training was shown to resolve the nemaline bodies formed during immobilization and reversed disuse-induced muscle atrophy and muscle weakness (60, 61). Therefore, the presence of nemaline bodies in SARS-CoV-2-associated myopathy is an important finding for the rehabilitation of COVID-19 patients. Functionally, NM patients are more prone to prolonged periods of immobility, which in turn exacerbates disuse-induced muscle atrophy and muscle weakness (62). Further research is necessary to investigate clinical outcome and/or functional recovery in critically ill patients infected with SARS-CoV-2 and to specify the training modality, intensity, and frequency needed to optimize skeletal muscle mass and recovery of muscle function in those patients. Furthermore, the significance and pathophysiological mechanism(s) of nemaline body formation in SARS-CoV-2-associated myopathy have to be elucidated.

Conclusions

In conclusion, the current electron microscopic study is the first to describe the disintegration of myofibrils and accumulation of Z-line material into nemaline bodies in skeletal muscle tissue obtained from critically ill COVID-19 patients following ICU admission. Since, a SARS-CoV-2 infection is known to induce a systemic cytokine storm, the myopathy observed in the COVID-19 patients can be considered as a necrotizing myopathy with nemaline bodies, in which the formation of nemaline bodies is a non-specific pathological reaction of extreme myofibrillar disintegration and thus a secondary epiphenomenon. The muscle fiber damage will potentially compromise clinical outcome and/or affect functional recovery. Our results are a call towards action for more research on clinical outcome and/or functional recovery and to develop and/or optimize specialized rehabilitation programs for COVID-19 patients.

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Tables

Table 1. COVID-19 Patients' characteristics

	Patient						
	1	2	3	4	5	6	7
Demographics and anthropometrics							
Age (years)	84	81	73	79	76	76	75
BMI (kg/m^2)	24.2	26.0	26.0	26.1	22.6	29.7	26.3
Gender (M:F)	M	M	M	M	M	M	M
Disease severity							
Acute Physiology and Chronic Health Evaluation II	17	7	10	16	12	11	20
Sequential Organ Failure Assessment score	9	15	6	11	8	7	12
Acute respiratory distress syndrome	yes	yes	yes	no	yes	no	Yes
Mode of ventilation	HFNO, NIV	NIV, IVM	NIV	NIV, IMV	HFNO, MIV	HFNO	IMV
Blood analysis							
White blood cells (x 10^9)	3.1	8.0	9.0	8.2	34.7	6.2	12.4
C-reactive protein (mg/L)	40	96	260	100	19	31	43
Lactate dehydrogenase (U/L)	530	640	430	320	450	240	370
Troponin (ng/L)	93	26.5	17.9	141	294	164	115
Comorbidities	CVD, DM	DM	Cancer, CVA	CVD, CRD	HTN	CVD, DM, CKD	CRD

HFNO: high flow nasal oxygen, NIV: non-invasive ventilation, IMV: invasive mechanical ventilation, CVD: cardiovascular disease, DM: diabetes mellitus, HTN: hypertension; CVA: cerebrovascular accident, CRD: chronic respiratory disease, CKD: chronic kidney disease

Figure legends

Figure.1. Electron micrographs of degenerative myofibers on admission (T0).

A) Normal striation pattern. B) Identifiable sarcomeres but lost normal striation pattern. C) Identifiable sarcomeres are absent. The myofibrillar disintegration is accompanied by smearing of the Z line material (white *) and involves few sarcomeres.

Figure 2. Electron micrograph of two adjacent longitudinally sectioned degenerative myofibers after 7 days of ICU stay (T1).

The myofibers show displaced triads (circles), Z line irregularities (arrow) and myofilament loss (white *). Insert A is a magnified image and shows part of the right/lower myofiber with preserved sarcomere structure, myofilament loss and swollen cisternae in the displaced triads. This part of the myofiber gradually transitions into a zone of severe myofibrillar disorganization, characterized by Z line irregularities involving several sarcomeres (insert B). The magnified image of the left/upper degenerated myofiber contains typical nemaline bodies, showing the same electron density as the Z-line material of the sarcomeres (insert C).

Figure 3. Thionin and methylene blue staining of araldite-embedded semithin sections.

A) Thread-like nemaline bodies (arrows) located at the periphery of longitudinally sectioned atrophic myofibers. B) Rod-like nemaline bodies located in the intermyofibrillar spaces (arrowheads) and in clusters (dotted ovals) at the periphery of a transversally sectioned myofiber.

Figure 4. Electron micrographs of degenerative myofibers without recognizable myofibrils after 7 days of ICU stay (T1), showing clusters of nemaline bodies.

A) Perinuclear cluster (oval) of parallel oriented thread-like nemaline bodies. Clustered nuclei (N). B) Subsarcolemmal transversely sectioned nemaline body (black arrow) and a cluster (circle) of chaotically distributed rod-like nemaline bodies in the centre of the myofiber, garlanded by accumulated mitochondria (M). Note that the central part of the largest nemaline bodies show cracks of low electron density (white arrowheads).

Figure 5. Electron micrograph of a degenerative atrophic myofiber after 7 days of ICU stay (T1).

The myofiber contains nemaline bodies (circle), clustered nuclei (N) with prominent nucleoli and local hypercontraction (white arrow) of myofibrils. Note the transition from a normal sarcomeric structure (white #) into myofibrillar disintegration (black *) and accumulation of Z-line material (white *), that is continuous with the nemaline bodies. Endomysium (E). Neighbouring capillary with thickened basal lamina (black arrow).

Figure 6. Electron micrographs of degenerative myofibers without recognizable myofibrils after 7 days of ICU stay (T1).

A) Myofilaments and desmin filaments (white *) were found associated with nemaline bodies. B) Some filaments (black *) anchor to the sarcolemma (black arrows). A neighbouring capillary (C) shows thickened and duplicated basal lamina accompanied by perivascular fibrosis (white #). Lipid droplet (Li). Glycogen (G). Dislocated triad

(circles). Mitochondrion (M) with swollen cristae. Endomysium (E). Note the crack of low electron density in the central part of a nemaline body (white arrowheads)

Figure 7. Electron micrograph of a degenerative myofiber without recognizable myofibrils after 7 days of ICU stay (T1).

B and C are magnified images of the boxed regions in A, showing subsarcolemmal nemaline bodies (circles) with typical lattice-like pattern. Mitochondria (M) with swollen cristae. Reduced continuity of membranes (black arrow). Clustered nuclei (N). Endomysium (E).