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**Diaphragmatic morphological *post-mortem* findings in critically ill COVID-19 patients:
an observational study**

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ABSTRACT

Our study investigates the *post-mortem* findings of the diaphragm's muscular structural changes in mechanically ventilated COVID-19 patients.

Diaphragm samples of the right side from 42 COVID-19 critically ill patients were analyzed and correlated with the type and length of mechanical ventilation (MV), ventilatory parameters, prone positioning, and use of sedative drugs. The mean number of fibers was 550 ± 626 . The cross-sectional area was $4120 \pm 3280 \mu\text{m}^2$, while the muscular fraction was 0.607 ± 0.126 . The overall population was clustered into two distinct populations (clusters 1 and 2). Cluster 1 showed a lower percentage of slow myosin fiber and higher fast fiber content than cluster 2, 68% *versus* 82%, $p < 0.00001$, and 29.8% *versus* 18.8%, $p = 0.00045$ respectively. The median duration of MV was 180 (41-346) hours. In cluster 1, a relationship between assisted ventilation and fast myosin fiber percentage ($R^2 = -0.355$, $p = 0.014$) was found. In cluster 2, fast fiber content increased with increasing the length of the controlled MV ($R^2 = 0.446$, $p = 0.006$). A high grade of fibrosis was reported. Cluster 1 was characterized by fibers' atrophy and cluster 2 by hypertrophy, supposing different effects of ventilation on the diaphragm but without excluding a possible direct viral effect on diaphragmatic fibers.

Key words: COVID-19, invasive mechanical ventilation, diaphragm dysfunction, myosin, ventilation-induce diaphragm injury.

Introduction

During spontaneous breathing, the main respiratory muscle, the diaphragm, generates a negative pressure permitting the air to penetrate the lungs and fill their space [1,2]. During non-invasive and invasive ventilation, the diaphragm can be partially assisted or fully supported during acute respiratory failure [3].

However, in animal models these supportive strategies resulted in oxidation of diaphragmatic proteins and increased diaphragmatic proteolysis due to elevated protease activity with consequent diaphragmatic damage. A selective down-regulation of myosin has been found to be concurrent with accelerated protein degradation by the ubiquitin–proteasome pathway [4].

Similarly, Levine et al. demonstrated in a small subset of 14 brain-injured patients that diaphragmatic atrophy during MV is sustained by activation of the ubiquitin-proteasome pathway [5].

Diaphragm atrophy and the potential onset of diaphragm weakness could culminate in ventilation-induced diaphragm dysfunction (VIDD) [6,7].

Other important risk factors associated with diaphragm weakness besides mechanical ventilation are the contemporary presence of sepsis, systemic inflammatory syndrome or the use of some medications, especially sedatives, steroids and muscle relaxant agents [8]. In non-COVID-19 patients, VIDD has a strong impact on clinical outcomes and is associated with difficult and prolonged weaning and extubation failure [9]. These consequences increase morbidity and mortality in critically ill patients [10].

It is unknown if critically ill COVID-19 patients who underwent mechanical ventilation experienced VIDD as the consequence of ventilation per se or as a direct effect of the virus as recently shown by Shi Z. et al. [11].

Moreover, a recent study evaluating 50 patients previously hospitalized with COVID-19 (14 females, age 58+12 years, half treated with mechanical ventilation and half treated outside intensive care settings with noninvasive ventilation) showed that diaphragm muscle weakness was still present 15 months after hospitalization. Furthermore, diaphragm weakness was associated with dyspnea on exertion. In this study, the authors identified diaphragm muscle weakness correlated with persistent dyspnea [12].

Therefore, our study aims to explore the anatomopathological diaphragm changes in COVID-19 ICU patients after mechanical ventilation.

Materials and Methods

Study protocol and design

This study was an observational post-mortem study of COVID-19 patients admitted to the Intensive Care Unit (ICU) who underwent invasive mechanical ventilation due to acute respiratory failure. The study was registered on ClinicalTrials.gov on January 13, 2022 (NCT05191433), after obtaining approval from the Institutional Review Board of the University of Udine (IRB ID 086/2021, November 22, 2021). This study follows STROBE reporting guidelines for observational studies.

Study population

Inclusion criteria were: i) adult mechanically ventilated patients aged 18 years and above; ii) deceased from SARS-CoV2 pneumonia in ICU who underwent post-mortem pathological examination. Exclusion criteria were: i) ventilated COVID-19 patients with a life expectancy < 48 hours; ii) COVID-19 positive patients admitted to the ICU with do-not-intubate or do-not-resuscitate orders; iii) severe end stage organ disease (patients awaiting liver or heart or kidney transplantation, patients under chronic hemodialysis); iv) extracorporeal membrane oxygenator support (ECMO).

Recorded patient data

Anthropometric data such as gender, age, weight, height and body mass index (BMI), previous medical history were recorded, as well as length of mechanical ventilation (MV) and length of stay in the ICU (ICU_{LOS}).

Pathology examination

All our institution's pathologists used the same protocol when performing post-mortem examinations of patients who died from SARS-CoV-2 infection. Diaphragm sampling was performed on the right side of the diaphragm at a distance of 2-3 centimetres from the costal insertion approximately 24 hours after the postmortem period. This area corresponds to the one explored with ultrasound and is known as the zone of apposition (ZOA). The portion of tissue obtained was then dissected and placed in Sakura Tissue-Tek Para form Sectionable Cassettes (Sakura Finetek Europe B.V., Mestre, Italy) according to various cutting planes to increase the probability of getting cross-sections of muscle fibres. The sample preparations were then processed, included in paraffin, and, from each block, sections were cut and later stained with hematoxylin-eosin. The resulting slides were scanned and acquired using a Leica Aperio AT2 slide scanner at 40× (Leica Biosystems, Milano, Italy).

Digital slides were then uploaded to the Omero Server (© 2005-2022 Glencoe Software, Inc.), where a pathologist annotated one or more regions of interest (ROI) per slide, choosing the points where the muscle fibres appeared to be cut transversely and with a rounded shape, avoiding picking those deformed or with an incorrect cutting plane (Figure 1 a,b).

Such regions were exported in TIFF format images for further data processing. Quantitative evaluation was carried out using a macro script for the ImageJ image analysis software (National Institutes of Health, USA, 2020). The muscle fibres were hand selected based on colour, excluding the smallest regions (possibly due to noise or non-muscle tissue). To evaluate the fibre sections, a filter based on the shape circularity was also used to discharge regions with more than one fibre (that result in an irregular and vast region) as shown in Figure 1c. For each ROI, the software calculated the number of fibres, the cross-sectional area (CSA), the mean perimeter and the mean diameter of the fibres, and the muscle fraction over the total tissue examined.

Immunohistochemical analysis

To define the fibre types, paraffin-embedded human skeletal muscle tissue labelling Slow Skeletal Myosin Heavy chain and Fast Myosin Skeletal Heavy chain +MYH4 was performed following Abcam antibody datasheet instructions (heat mediated antigen retrieval with Tris/EDTA buffer pH 9.0, use of clone ab234431 or clone ab255685, respectively, at 1/500

dilution), followed by a ready-to-use Rabbit specific IHC polymer detection kit HRP/DAB. The section was incubated with ab234431 for 30 mins at room temperature. The immunostaining was performed on a Ventana instrument and counterstained with hematoxylin. Cytoplasmic positivity on the human skeletal muscle was obtained as shown in Figure 1d. Antibodies directed against slow and fast were performed in two consecutive slides, therefore distant approximately 3,5-4 μm from each other, a distance that could hardly differentiate significantly the fiber morphology, allowing us to superimpose the two slides.

Ventilation parameters and intensive care unit treatments

We reported mechanical ventilation mode (pressure-controlled, volume-controlled, volume-guaranteed pressure-controlled or pressure support ventilation [PSV]), ventilation parameters (tidal volume [V_T], plateau pressure [P_{plat}], PEEP, pressure support [PS], F_{IO_2}) and blood gas analysis results (PaO_2 , PaCO_2 , $\text{PaO}_2/\text{F}_{\text{IO}_2}$ ratio). The respiratory system driving pressure (ΔP), elastance (E_L) and mechanical power (MP) according to Guerin's method were computed whenever possible as following: $0.098 \times V_T \times \text{RR} \times \Delta P$ [13]. Moreover, we used the recent simplified formula as suggested by Costa [$(4 \times \Delta P) + \text{RR}$] that is as informative as mechanical power [14].

Hemodynamic parameters (HR and mean arterial pressure [MAP]), vasopressors use, sedation, muscle relaxants, pronation, and nitric oxide use were also recorded.

Study outcomes

This study investigates the histopathologic post-mortem findings of the diaphragm in critically ill COVID-19 patients who underwent mechanical ventilation. As a secondary outcome, we investigated possible clusters of patients defined by different characteristics in pathology findings, if any. We finally explored potential correlations between pathology findings and the different modes of mechanical ventilation, prone position, iNO administration, neuromuscular blockers use and the effects of the sedative drugs.

Statistical analysis

Continuous variables are expressed as mean and standard deviation or median and minimum/maximum values or interquartile ranges where appropriate. Categorical variables are expressed as absolute numbers and percentages. The Student t-test or the Wilcoxon-Mann-Whitney test was used to compare continuous variables, based on Kolmogorov-

Smirnov normality test results, and the χ^2 analysis or the Fisher exact test for categorical variables, as appropriate based on expected frequencies.

We used the K-medoids clustering PAM algorithm (Partitioning Around Medoids) to cluster our population, including in the algorithm the three most important anatomic-pathological variables (fast myosin, slow myosin and the number of fibres). We used the average silhouette method to estimate the optimal number of clusters. The Wilcoxon-Mann-Whitney test was used to compare continuous variables between the two clusters, and the Fisher exact test was used to compare categorical variables between the two clusters. All these analyses were done in the overall population. However, to be more precise and to evaluate the potential relationship (with Pearson or Spearman's correlation test as appropriate) between anatomopathological findings and ventilatory parameters, we decided to include only patients with complete data regarding ventilatory parameters.

The sample size was calculated considering an expected difference of 500 μm^2 in CSA between group 1 and 2 as the main objective.

Assuming a pooled standard deviation of 470 μm^2 , we have calculated a sample size of 14 for each group (i.e. a total sample size of 28, assuming equal group sizes), to achieve a power of 80% and a level of significance of 5% (two sided), for detecting a true difference in means between group 1 and 2 of 500 (i.e. 3000 - 2500) μm^2 .

All statistical analyses were performed using R software version 4.0.2 (R Core Team 2020, Vienna, Austria). Figures reporting correlations were made with the GraphPad Prism version 9.4.1. The significance level was set at 0.05. No imputation was done for missing data.

Results

From November 1st, 2020, to March 30th, 2021, we studied 42 patients. They were mainly men (83%), 70 [66-76] years old, with a BMI of 27.8 [25.4-31.2] Kg/m^2 . Previous medical history is furnished as *Supplementary Table 1*.

Median duration of MV was 180 [41-346] hours. Moreover, median ICU length of stay (ICU_{LOS}) was 240 hours [96-396], corresponding to 10 days.

The overall population showed a higher percentage of slow than fast fibres, 74.4% and 24.8%, respectively. The mean number of fibres in the ROIs investigated was 550 ± 626 . The Cross-Sectional Area (CSA) of fibres was $4120 \pm 3280 \mu\text{m}^2$. Mean perimeter was $274 \pm 111 \mu\text{m}$, while maximum diameter was $93 \pm 37.8 \mu\text{m}$. The muscular fraction (area occupied by muscle fibres over total area analyzed) was 0.607 ± 0.126 .

According to the partition analysis described in the methods section, we detected two clusters, dividing the population into Cluster 1 (23 patients) and Cluster 2 (19 patients). There

were no significant differences in the baseline characteristics of the two clusters, as shown in *Supplementary Table 1*. They differed in the percentage of slow and fast myosin, and the number of fibres in the ROIs investigated, as shown in Table 1.

When analyzing ventilation data, we included 29 patients, 14 belonging to Cluster 1 and 15 in Cluster 2.

A trend toward statistically significant difference was observed when comparing Cluster 1 vs Cluster 2 regarding the percentage of time spent under controlled mechanical ventilation and sedation during ICU stay, 59% vs 53%, $p=0.06$, and 92% vs 81%, $p=0.07$, respectively, as shown in Table 2.

As reported in Table 3, V_T , ΔP and E_L of the respiratory system were similar in Cluster 1 and 2 ($p=0.90$ for tidal volume, $p=0.07$ for ΔP and $p=0.11$ for E_L). On the contrary, MP was lower in Cluster 1 than Cluster 2, 17.2 versus 21.4 J/min ($p=0.03$) respectively. Similarly, the results of Costa's formula were 95 versus 107 in Cluster 1 and Cluster 2 respectively ($p=0.009$).

A weak positive correlation was observed in the overall population between the time spent under controlled mechanical ventilation and the percentage of fast myosin fibres ($R^2=0.156$, $p=0.034$). Similarly, the longer the time under pronation, the higher the muscle fibres' fraction ($R^2=0.197$, $p=0.017$).

Analyzing the relationship between ventilatory data and anatomopathological characteristics in the two clusters separately, we found a negative relationship between the time spent under assisted ventilation and the percentage of fast myosin fibres ($R^2=-0.355$, $p=0.014$) in Cluster 1. On the contrary, Cluster 2 showed a direct positive linear relationship between the same parameters ($R^2=0.246$, $p=0.059$). In Cluster 2, the percentage of fast fibres increased with increasing the amount of time spent under controlled mechanical ventilation ($R^2=0.446$, $p=0.006$). Number per micron was reduced for prolonged periods of controlled mechanical ventilation ($R^2=-0.34$, $p=0.022$) (*Supplementary Figure 1*). No correlation was found for the same variables in cluster 1. A negative correlation between the number of muscular fibres and the total length of mechanical ventilation ($R^2=-0.293$, $p=0.037$) was found in Cluster 2.

Inverse correlation between mean tidal volume and the percentage of fast myosin fibres was found in Cluster 1 ($R^2=-0.37$, $p=0.022$). On the contrary, the same correlation was positive in Cluster 2 ($R^2=0.312$, $p=0.046$) as shown in Figure 2. A direct positive correlation between the P_aO_2/F_iO_2 ratio and the number of fibres was found only in Cluster 1 ($R^2=0.514$, $p=0.004$). Mechanical power demonstrated a negative linear correlation with the percentage of fast fibres in Cluster 1 ($R^2=-0.459$, $p=0.031$). MP and Costa's formula demonstrated a positive linear correlation ($R^2=0.67$, $p<0.0001$). All comparisons between ventilatory and anatomopathological correlations are available as *Supplementary Table 2*.

Discussion

The main findings in the present study are i) in mechanically ventilated critically ill COVID-19 patients, slow muscular fibres are predominant over fast fibres; ii) extracellular components represented 40% of ROIs investigated; iii) based on anatomopathological findings, two distinct clusters of patients could be described, where Cluster 1 is characterized mainly by atrophy of the diaphragm, and Cluster 2 is characterized by hypertrophy of diaphragmatic muscular fibres; iv) there was a different relationship between anatomopathological findings and ventilation features within the clusters; in particular in Cluster 1, fast fibres decreased with increased time under assisted ventilation and tidal volume; in Cluster 2, a positive correlation was found between fast fibre percentage and time under assisted ventilation and tidal volume.

Slow and fast fibres are usually in about equal proportions in the adult human diaphragm (about 55% slow fibres, 45% fast fibres) [15].

Jaber et al., in a small cohort of non-COVID critically ill patients after long-term (80 hours) and short-term (2 hours) mechanical ventilation for endoscopic digestive procedures with general anaesthesia, reported a preserved type 1 over type 2 fibre ratio close to 50% [6].

In our study, on the contrary, about 74% of fibres were represented by slow type (type I).

The unusually high percentage of fast MHC positive fibers in comparison with the typical observation in non COVID-19 patients could be explained as a response to the increased neural respiratory drive observed in these patients, which in turn increases the protein synthesis to sustain the work of breathing [16-18].

We observed, also, an increased quote of fibrosis that reached about 40% of the tissue examined since it usually occupies a proportion between 1-20% of human skeletal muscle [19].

Our results seem to confirm a previous study in COVID-19 ICU patients by Shi Z et al. [11] In postmortem diaphragm findings from three medical centres they observed an increased expression of genes involved in fibrosis and histological evidence for the development of fibrosis in the diaphragm. This “myopathic phenotype” was different from that of control-ICU patients, considering comparable duration of mechanical ventilation and ICU length of stay. The authors could not establish whether diaphragm myopathy is a direct effect of SARS-CoV-2 [11].

However, it is probable that not all patients develop the same diaphragmatic alterations. In fact, we identified two different clusters of patients considering the anatomopathological findings: Cluster 1 showed atrophy of the diaphragm, while Cluster 2 patients were more likely to show hypertrophy.

The structural and functional characteristics of respiratory muscle fibres are not fixed, and can be modified in response to several physiological and pathological conditions such as gender, training, age related modifications, changes associated with pharmacological agents (α 2-agonists and corticosteroids for example) or hypoxia [19].

Training-induced hypertrophy increases the muscle fibre cross-sectional area (f-CSA) and would result in a proportional increase in myofibrillar protein abundance, reported to happen through the addition of sarcomeres in series or parallel in existent myofibrils (i.e., sarcomerogenesis), or due to the synthesis of new myofibrils in existent muscle fibres (i.e., myofibrillogenesis) [20].

Given the composition of skeletal muscle, patients in Cluster 2 probably developed a different kind of hypertrophy, not only caused by an increase in sarcomeres or myofibrils but also by connective tissue hypertrophy (due to an increase in the volume of the extracellular matrix that shows an increase in mineral or protein) [21].

One consideration should be explored at this point: it is possible that COVID-19 patients experience lengthy periods of silent hypoxemia with low arterial oxygen content stimulating a high respiratory drive to increase oxygen delivery [22,23]. This can be considered an endurance-like stimulus during respiratory muscle recruitment, which could favour type 1 over type 2 fibres, considering the former are more suitable for sustained prolonged workloads [24].

It has been shown that during controlled MV, the right and left hemidiaphragms undergo passive shortening during mechanical expansion of the lungs. In contrast, bilateral inactivity of the diaphragm, due to either nerve blockage or denervation, results in passive stretching of the inactivated diaphragm region during inspiration. This is significant within our context because it has been postulated that the transient hypertrophy of Type 1 and 2a fibers in the rat diaphragm during bilateral denervation is due to increased protein synthesis induced by the passive stretch of the muscle during breathing [4,25]. Shortening on diaphragmatic adaptation to inactivity remains unknown; this is an interesting area for future research.

Moreover, a high respiratory rate induces high respiratory loading which is known to cause diaphragm myofibre injury [26].

In other words, critically ill COVID-19 patients appear more prone to developing fibrosis due to the direct stimulation of enzymes involved in the fibrogenic pathway which could impair diaphragmatic performance [27]. However, appropriate respiratory support during pandemic was guaranteed only in part due to the scarcity of ventilators and hospitals reorganization [28].

Investigation of the diaphragm's function with ultrasound has been increasingly used [29,30].

Cammarota et al. have recently shown that the pronation of COVID-19 patients with C-ARDS can increase the diaphragmatic thickening fraction. The authors explain this process through the increased respiratory workload induced by the prone position, which is determined by reduced chest wall compliance [31]. In agreement with this hypothesis, we observed a positive linear correlation between the muscle/total tissue ratio increase and time spent in the prone position in our COVID-19 patients in Cluster 2.

In addition, the number of fibres in the same cluster (cluster 2) was reduced by increasing the duration of controlled mechanical ventilation. These results confirm that prolonged mechanical ventilation induces atrophy in the diaphragm [32].

We do not know if this change is directly affected by SARS-CoV-2 [12] or whether it is due to mechanical ventilation per se.

Similar results have been found by Hennigs et al. in a study where they described respiratory muscle dysfunction five months after COVID-19 infection across all the spectrum of the disease, from mild to severe patterns [33].

These works are another piece of the puzzle about the complex interplay between possible direct effect of the virus on the diaphragm, and injury caused by mechanical ventilation.

Interestingly, in our study assisted ventilation (pressure support) and the percentage of fast fibres demonstrated a very different correlation in Cluster 1 compared to Cluster 2. While in the former, the fast fibre percentage showed a reduction for longer assisted ventilation periods and higher V_T , an inverse correlation was found in the latter cluster.

MP demonstrated a negative linear correlation with the percentage of fast fibres in Cluster 1, however Costa's formula did not. As such, we argue that V_T , which was the only different variable between them, could play a possible role in determining this change in diaphragm structure.

Mechanical ventilation per se alters diaphragm structure. However, the changes in the structural composition of respiratory muscles such as the diaphragm are probably due to multifactorial events: both the increase of the respiratory workload and the inflammation (during early phase) and the diaphragmatic dysfunction caused by mechanical ventilation, fibrogenesis processes (in the late phase) could be involved as common causes, leading to poor residual lung function [34,35]. Further genetic assessments related to the expression of Type I and Type II muscle fibres in critically ill COVID-19 patients' diaphragms might be useful.

This is an exploratory study aimed at evaluating changes induced by mechanical ventilation in the diaphragmatic musculature of critically ill COVID-19 patients. Our study carries some limitations. First, the limited sample size precludes any generalization of the results.

We have not evaluated additional variables that may be involved in the determinism of the changes mentioned above. In addition, we cannot assess the impact of different ventilation modes and settings beyond on the observed changes in diaphragm structure. In addition, we cannot exclude a direct effect of virus on diaphragmatic fibres since no comparison group (non COVID-19 patients) has been studied.

Some limitations of CSA calculations by the microscopic assessment due to the method of tissue processing (i.e. climate of the laboratory, buffers, etc.), biopsy location (it is challenging to biopsy the exact location in a muscle) and measurement methods need also to be acknowledged. Moreover, the muscle can respond to training in a non-uniform manner (proximal versus distal), which is difficult to appreciate in a single-site measurement.

Finally, we did not investigate the molecular pathway potentially related to gene expression, such as fibrogenic or hypertrophic pathways. However, this was beyond the aim of this study.

Conclusions

In mechanically ventilated critically ill COVID-19 patients, fast myosin fibres were reduced, and extracellular components increased with a net prevalence of slow fibres. This led us to describe two patient clusters: one in which atrophy prevailed and a second cluster in which hypertrophy was most prevalent. The mechanical power instead of ΔP and the formula $(4 \times \Delta P) + RR$ were able to capture this ventilation-diaphragm interaction.

These results need confirmation in prospective studies with larger sample size also in patients without COVID-19.

List of abbreviations:

COVID-19=coronavirus disease-19; VIDDD=ventilation-induced diaphragm dysfunction; ICU=intensive care unit; ECMO=extracorporeal membrane oxygenator support; BMI=body mass index; ICU_{LOS}=length of stay in the ICU; ZOA= zone of apposition; ROI= regions of interest; CSA= cross-sectional area; V_T=tidal volume; P_{plat}=plateau pressure; PEEP=end-expiratory positive pressure; ΔP =driving pressure; E_L=elastance; MP=mechanical power; RR=respiratory rate; HR=heart rate; MAP=mean arterial pressure; DTF= diaphragm thickening fraction.

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Online supplementary material:

Supplementary Figure 1. Fast fiber content and their number relationship with mechanical ventilation. Fast myosin content showed a positive linear correlation with the percentage of controlled mechanical ventilation on total LOS_{ICU} ($R^2=0.446$, $p=0.006$ as shown in panel A). On the opposite, their number decreased regarding the same ventilation parameters ($R^2=-0.34$, $p=0.022$ as shown in panel B).

Supplementary Table 1. Pre-existing conditions and relevant medical history.

Supplementary Table 2. Correlations between ventilatory parameters and anatomopathological findings.

Table 1. Biopsies findings and comparison between clusters.

	OVERALL (n=42)	CLUSTER 1 (n=23)	CLUSTER 2 (n=19)	<i>p-value</i>
Slow myosin %	74.4 (9.7)	68.3 (7.01)	81.8 (6.91)	<i><0.00001</i>
Fast myosin %	24.8 (10.2)	29.8 (7.30)	18.8 (10.2)	<i>0.00045</i>
N° fibers	550 (626)	790 (756)	259 (182)	<i>0.00030</i>
CSA (μm^2)	4120 (3280)	3380 (2780)	5010 (3680)	0.07675
Perimeter (μm)	274 (111)	248 (101)	305 (118)	0.06850
Diameter (μm)	93.0 (37.8)	84.4 (33.6)	103 (40.8)	0.12407
Muscle fraction	0.607 (0.126)	0.604 (0.141)	0.611 (0.108)	0.94953

Values are expressed as mean and standard deviation. CSA, cross sectional area.

Table 2. Main intensive care unit data.

	OVERALL (n = 29)	CLUSTER 1 (n = 14)	CLUSTER 2 (n=15)	p-value
Duration of MV (hours)	180 [41-346]	231 [102-425]	167 [8-295]	0.27
NIV (% over total time of ICU_{LOS})	0 [0-0.04]	0 [0-0.01]	0.01 [0-0.1]	0.23
Controlled MV (% over total time of ICU_{LOS})	0.57 [0.30-0.70]	0.59 [0.45-0.81]	0.53 [0.17-0.59]	0.06
PSV (% over total time of ICU_{LOS})	0.12 [0-0.27]	0.08 [0.03-0.31]	0.18 [0-0.28]	0.70
Sedation (% over total time of ICU_{LOS})	0.88 [0.52-0.94]	0.92 [0.77-0.96]	0.81 [0.21-0.93]	0.07
Muscular Paralysis (% over total time of ICU_{LOS})	0.18 [0-0.43]	0.14 [0.025-0.42]	0.27 [0-0.43]	0.74
Prone positioning (% over total time of ICU_{LOS})	0.04 [0-0.12]	0.09 [0-0.14]	0.03 [0-0.11]	0.36
iNO (% over total time of ICU_{LOS})	0 [0-0.15]	0 [0-0.20]	0 [0-0.17]	0.98
PaO₂ (mmHg)	75 [66 - 91]	73.5 [66-90]	77 [66 - 91]	0.74
PaCO₂ (mmHg)	45 [39 - 54]	44 [38 - 51]	48 [43 - 66]	0.11
PaO₂/F_IO₂ (mmHg)	128 [100-178]	136 [93-257]	115 [100-145]	0.34
MAP (mmHg)	77 [68 - 90]	82 [70 - 93]	71 [65 - 84]	0.10
HR (bpm)	71 [60-87]	64 [50-74]	74 [70-100]	<i>0.001</i>

Data are expressed as median and [interquartile ranges]. MV, invasive mechanical ventilation; ICU, intensive care unit; NIV, non invasive ventilation; PSV, pressure support ventilation; ICU_{LOS}, ICU length of stay; iNO, nitric oxide; MAP, mean arterial pressure; HR, heart rate.

Table 3. Ventilation parameters.

	OVERALL (n = 29)	CLUSTER 1 (n = 14)	CLUSTER 2 (n=15)	p-value
P_{plateau} (cmH₂O)	34 [30-35]	32 [28-34]	34 [30-36]	0.15
PEEP (cmH₂O)	12 [10-14]	12 [10-14]	12 [10-14]	0.76
PS (cmH₂O)	12 [10-13]	12 [10-13]	12 [10-14]	0.80
F_iO₂ (%)	60 [50-80]	55 [35-65]	70 [60-80]	<i>0.02</i>
RR (apm)	20 [17-21]	20 [20-23]	18 [19-22]	0.07
V_T (mL/Kg IBW)	6.8 [6.2-7.6]	6.8 [6.2-7.6]	6.7 [6.2-8]	0.90
ΔP (cmH₂O)	20 [18-22]	19.5 [17.7-21.0]	22 [20-24]	0.07
E_L (cmH₂O/L)	38.2 [35.3-41.5]	37.8 [34.5-39.5]	39.3 [35.9-46.2]	0.11
MP (J/min)	20.15 [16.1-22.8]	17.2 [13.8-20.6]	21.4 [17.9-26.1]	<i>0.03</i>
Costa's formula [(4 x ΔP) + RR]	100 [94.5-107.5]	95 [86.7-101.8]	107 [98-114]	<i>0.009</i>

Data are expressed as median and [interquartile ranges]. P_{peak}, peak pressure; PEEP, end expiratory positive pressure; RR, respiratory rate; V_T, tidal volume; IBW, ideal body weight; ΔP, driving pressure; E_L, elastance; MP, mechanical power.

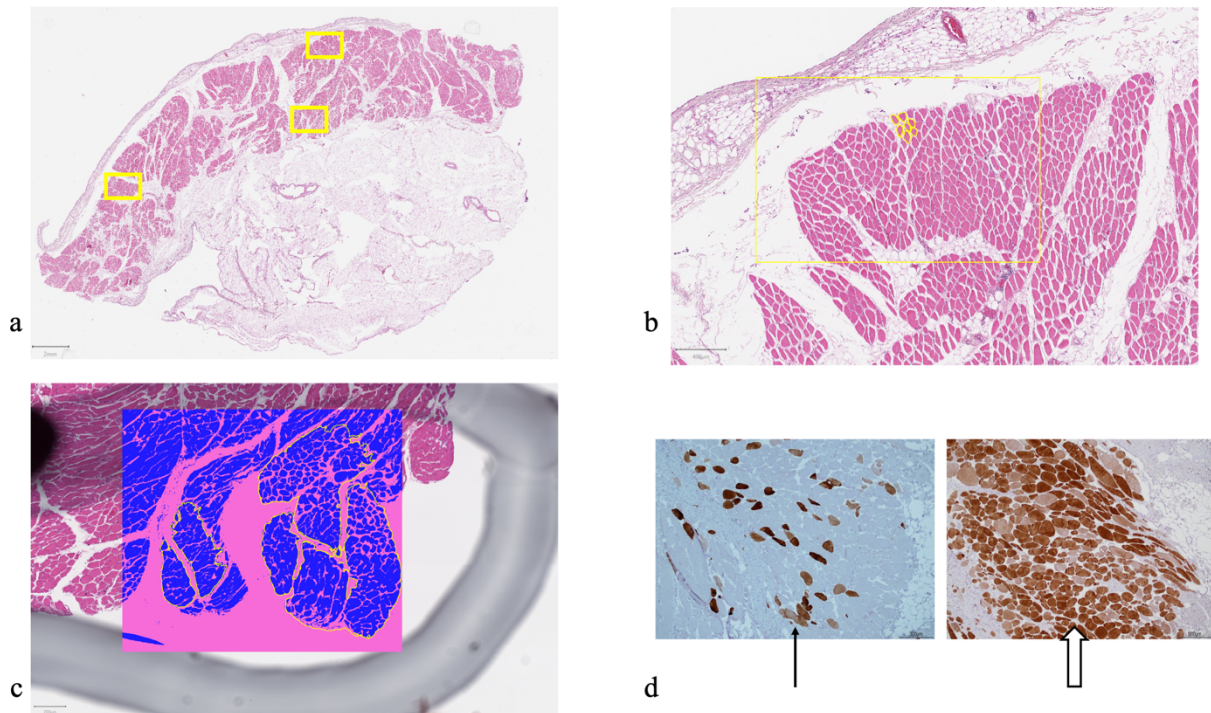


Figure 1. Diaphragm sections and digital acquisition of images. The diaphragm tissue slides were scanned and acquired using a Leica Aperio AT2 slide scanner at 40x. Digital slides were then uploaded in a Omero Server, where a pathologist annotated one or more regions of interest per slide (ROIs) as shown in figure 1a. After that, the pathologist annotated the points where the muscle fibers appeared to be cut transversely and with a rounded shape, avoiding picking those that appeared deformed or with incorrect cutting plane (figure 1b). Quantitative evaluation has been carried out by means of a macro script for the ImageJ image analysis software (figure 1c). The script selected the muscle fibers basing on color, excluding the smallest regions (possibly due to noise or non muscle tissue). For evaluating the fiber sections, a filter based on the shape circularity has been also used, to discharge regions constituted by more than one fiber (that result in an irregular and very large region). Thin arrow shows slow skeletal myosin heavy chain cytoplasmic positivity (Abcam clone ab234431, 4x), while black empty arrow points put fast skeletal myosin heavy chain cytoplasmic positivity (Abcam clone ab255685, 4x) in figure 1d. Antibodies directed against slow and fast were performed in two consecutive slides, therefore distant approximately 3,5-4 μm from each other, a distance that could hardly differentiate significantly the fiber morphology, allowing us to superimpose the two slides.

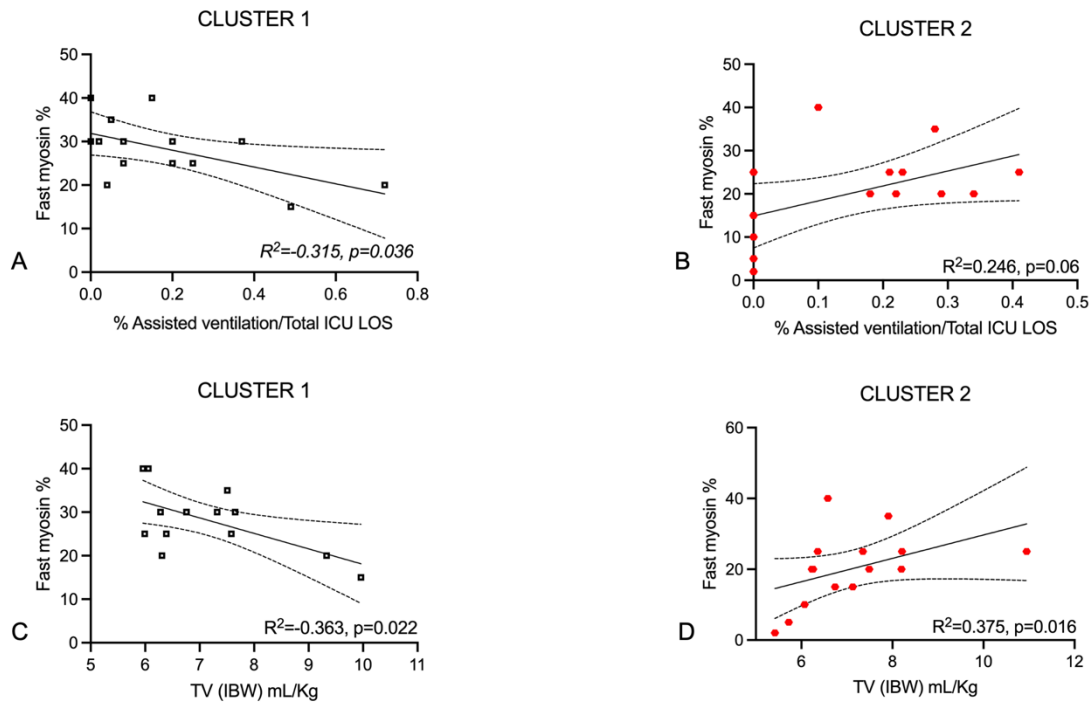


Figure 2. A) Fast fiber content relationship and ventilatory parameters within the two different clusters. Negative relationship between the time spent under assisted ventilation and the percentage of fast myosin fibres ($R^2 = -0.355$, $p = 0.014$) was found in Cluster 1; B) on the contrary, Cluster 2 showed a direct positive linear relationship for the same parameters ($R^2 = 0.246$, $p = 0.059$); C) an inverse correlation between mean tidal volume and the percentage of fast myosin fibres was highlighted in Cluster 1 ($R^2 = -0.37$, $p = 0.022$); D) on the contrary, the same correlation was positive in Cluster 2 ($R^2 = 0.312$, $p = 0.046$).